Sterol production in yeast

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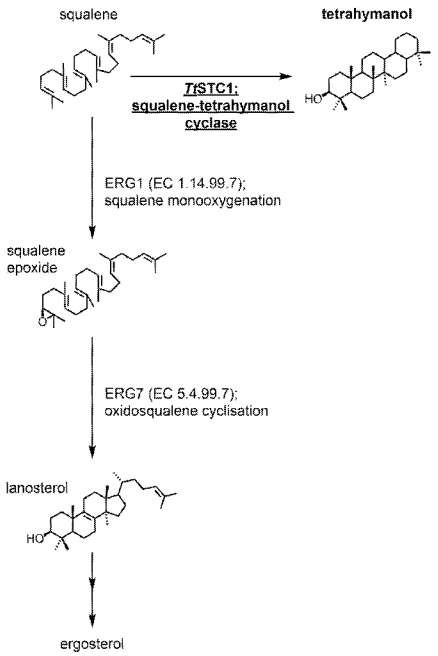
Figure 3



(57) Abstract: The present invention relates to genetically-modified oleaginous yeasts for producing non-native sterols at commercially useful levels, especially for example in providing sterols individually or as a mixture in an artificial dietary composition for honeybees or other insects or animals. For this purpose, an oleaginous yeast, e.g. *Yarrowia lipolytica*, may be employed wherein the yeast has reduced production of ergosterol compared with a wild-type oleaginous yeast or is incapable of producing ergosterol and is provided with a sterol surrogate to aid growth. From such yeast, however, other yeast may be engineered which retain useful sterol production without need for a sterol surrogate, e.g. production of sterol mixtures in which 24-methylenecholesterol or campesterol is the dominant sterol.

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Sterol Production In Yeast

Field of Invention

The invention relates to methods for producing sterols and compounds derived therefrom. In particular, the invention relates to genetically modified yeasts, in particular oleaginous yeasts for producing non-native sterols endogenously and methods of using such yeasts. Non-native sterols and non-native sterol mixtures produced as now disclosed have uses in a wide variety of compositions including artificial dietary compositions, food products and pharmaceutical compositions. One application of sterols and sterol mixtures produced as now taught and which is of especial interest is in provision of artificial dietary compositions for honeybees. Honeybees are sterol auxotrophs and hence phytosterols such as 24-methylenecholesterol are essential nutrients for such bees. However, up to now no method has been available for readily commercially producing sterols for this purpose by culturing cells such as yeast cells using a cheap and simple carbon source.

Background

Sterols are a subset of triterpenoids ubiquitous among eukaryotes. Their biological functions are diverse. Notably, sterols are structural components of phospholipid membranes, steroidal hormone precursors and signalling molecules. Terminal intermediates in the phytosterol biosynthesis pathway, including desmosterol, cholesterol, 24-methylenecholesterol, campesterol, isofucosterol, beta-sitosterol and stigmasterol, are often the most prevalent sterols in plants. For plants, sterols may be used for growth, development regulation and hormone synthesis as well as pest and pollinator control. Sterols may be used in the formation of artificial food compositions that replace natural sterol sources, such as a pollen replacement for pollinators such as bees and other insects.

For example, the domesticated insect, the Western honeybee (*Apis mellifera*) requires a mixture of sterols. Svoboda et al. (1980) J. Insect Phys. 26, 291-294 and Svoboda et al. (1986) Insect Biochem. 16(3): 479-482) teach that honeybees' tissues are comprised of a mixture of sterols derived from floral pollen that include 24-methylenecholesterol (51.2%), β-sitosterol (23.7%), isofucosterol (13.2%), campesterol (8.7%), cholesterol (2.2%), and desmosterol (1.0%); the relative proportions of each sterol can vary from 5-10%. Svoboda et al. (1979) J. Insect Phys. 26: 287-289 further teaches that when bees are deprived of any of these sterols, the whole colony will die within 8-12 weeks, thus demonstrating the essentiality of these particular sterols in diet and the essentiality of their plurality.

Published International Application WO2017/085477 (University of Newcastle-Upon-Tyne) relates to provision of artificial bee feed comprising 24-methylenecholesterol, e.g. bee feed comprising 24-methylenecholesterol and one or more other phytosterols such as campesterol, β-sitosterol and cholesterol. However, some phytosterols, including 24-methylenecholesterol and isofucosterol are not produced industrially. For this reason, they cannot be readily provided to bees or other domesticated insects in feed at the present time. As indicated above, there has remained a need for preparing such a composition by means of convenient yeast cell culture, especially using an oleaginous yeast. Oleaginous yeasts such as *Yarrowia lipolytica* have high capacity for sterol ester storage in lipid particles and are more suitable for non-native sterol production than for example *Saccharomyces*

cerevisiae. Nevertheless, *S. cerevisiae* has been much used in investigation of enzymic pathways in both yeast and plants for sterol production. Such studies however, as further discussed below, have not enabled production of sterols and sterol compositions as now taught and desired for many applications, including bee feeds.

Sterols, such as phytosterols, are of further industrial interest, as precursors to brassinosteroids. Brassinosteroids have agricultural application as plant growth hormones, as well as broad pharmaceutical applications.

Sterols, such as phytosterols, have a cholesterol-lowering effect when present in the diet of mice and humans, via inhibitory effects on cholesterol absorption in the liver. Plant sterols are advised as part of a healthy diet for the management of dyslipidemia. They may be desirably added to a variety of food products.

Phytosterols have also been associated with reduced risk of cancer, cardiovascular disease, arthritis, hepatic inflammation, and may have anti-oxidant function. Phytosterols are of further pharmaceutical interest, as precursors to withanolides. These bioactive steroidal lactones are of broad pharmacological interest, including the treatment of cancers, inflammation and ageing. Similarly, sterols and phytosterols are precursors to important steroidal compounds. For example, cholesterol can be used for the synthesis of vitamin D3.

24-methylenecholesterol is not presently available commercially. Several other phytosterols (e.g. β-sitosterol) can currently be isolated from vegetable oils such as wheatgerm oil. However, phytosterols are present at very low abundance in plant tissue, making isolation of phytosterols by extraction non-viable on a commercial scale. Plant sources also contain a mixture of phytosterols which are difficult to separate due to their highly similar structures and chemical properties. Cholesterol is commonly obtained by isolation from lanolin and cattle spinal cord. As for plant tissues, yield is low, and supply is further limited by extraction efficiency. Total chemical synthesis of cholesterol has been described, for example from hydroquinone. However, this is complex and inefficient for industrial production of phytosterols. Further, stereospecificity, particularly of C-24 ethyl sterols, is difficult to achieve.

Consequently, modified yeast strains have been of interest for producing non-native phytosterols. Endogenous sterol pathway flux in yeast is such that only the end-product sterol, ergosterol, accumulates. All preceding intermediates are transient and represent a small percentage of the overall sterol component of the cell. Since the fungal sterol biosynthesis pathway shares intermediates with those of plants and mammals, yeast cells have been engineered to divert sterol pathway flux away from ergosterol toward an alternative, non-native sterol. Production of desmosterol, cholesterol, 24-methylenecholesterol, campesterol, isofucosterol and β-sitosterol has been achieved by introducing varying combinations of heterologous sterol reductases and sterol methyltransferases, whilst inactivating endogenous ergosterol pathway genes encoding Erg4p, Erg5p or Erg6p.

The sterol composition of *Saccharomyces cerevisiae* has been modified by altering the activity of enzymes involved in ergosterol synthesis; enzymes in the latter half of the pathway are non-essential. Reduced oxygen or heme availability inhibit ergosterol synthesis, since the pathway is metabolically

demanding and oxygen-dependent. Under such conditions, *S. cerevisiae* will take up diverse sterols from the growth medium to substitute ergosterol function. This ability is not common in other yeast genera: most yeasts lack sterol uptake transporters and therefore, the genes encoding enzymes necessary in the final steps of sterol production may be considered essential.

A method for the production of cholesterol in *Saccharomyces cerevisiae* has been described in Souza et al. (2011) Metabolic Eng. *A stable yeast strain efficiently producing cholesterol instead of ergosterol is functional for tryptophan uptake, but not weak organic acid resistance*. The strategy to create the cholesterol-producing strain involved disrupting the ERG5 and ERG6 genes and replacing them with genes encoding dehydrocholesterol reductases DHCR24 and DHCR7 from fish.

Sawai et al. (2014) The Plant Cell, St*erol side chain reductase 2 is a key enzyme in the biosynthesis of cholesterol, the common precursor of toxic steroidal glycoalkaloids in potato*, discloses an engineered *S cerevisiae* yeast strain in which both the ERG4 and ERG5 genes have been knocked out and in which a potato (*Solanum tuberosum)* gene has been introduced for expression of the delta-7 sterol reductase, StDWF5. However, the sole interest of the authors is elucidating the biosynthetic pathway of cholesterol and related steroidal glycoalkaloids and the content or purity of 24-methylenecholesterol was not quantified. They do not address producing 24-methylenecholesterol for commercial use with a cheap carbon source or indeed teach whether this is feasible.

A method for the production of campesterol in *S. cerevisiae* has been described in Tsukagoshi et al. (2016) J. Biol. Chem. *Ajuga delta 24-sterol reductase catalyzes the direct reductive conversion of 24-methylenecholesterol to campesterol*. They employed the above-noted engineered *S. cerevisiae* strain of Sawai et al. solely to characterise the ArDWF1 gene from an expression sequence tag library of *Ajuga reptans* var. *atropurpurea* and compare this with *Oryza sativa* DWF1. They established that ArDWF1, like OsDWF1, is functionally equivalent to yeast Erg4p and resulted in production of campesterol. However, again the studies did not address producing 24-methylenecholesterol nor suggest how 24-methylenecholesterol might be usefully produced in any engineered yeast strain in useful quantity for commercial use.

Oleaginous yeasts such as *Yarrowia lipolytica* and *Rhodotorula sp*. have the capacity to grow on lipidic substrates such as vegetable oils. This provides the potential for growth on waste vegetable oils: a cheap and abundant carbon source with a positive environmental impact.

Campesterol production in *Yarrowia lipolytica* is described in CN107083338A. Campesterol production in *Y. lipolytica* using glucose or sunflower seed oil as the carbon source has also been described in Du et al. (2016) PLOS ONE, *Engineering Yarrowia lipolytica for campesterol overproduction*, Zhang et al. (2017) Biotechnol. Lett. *Improved campesterol production in engineered Yarrowia lipolytica strains,* and Qian et al. (2020) Appl. Microbiol. Biotech. *Increased campesterol synthesis by improving lipid content in engineered Yarrowia lipolytica*. For such campesterol production, it was found possible to knock out the ERG5 gene of the *Y. lipolytica* cells employed and express in the same cells a codon-optimised coding sequence for a 7-dehydrocholesterol reductase (DHCR7). The DHCR7 gene from *Xenapus laevis* or *Danio rerio* was favoured.

Such studies do not consider, however, the stereochemistry around the C-24 which can be expected to be affected by reliance on endogenous yeast ERG4 for conversion of 24-methylenecholesterol to campesterol; it can be expected that an epimer of plant campesterol will be produced [24β configuration (S) rather than the correct plant 24α configuration (R)]. As discussed in Xu et al. (2020) ACS Synth. Biol. *Engineering of phytosterol-producing yeast platforms for functional reconstitution of downstream biosynthetic pathways,* for many applications, including downstream synthesis, it will be desirable to retain the stereochemistry at the C-24 of campesterol to be expected from use of a plant DWF1 delta-24 sterol reductase (see Figure 16 of the present application).

Xu et al. hence used only plant enzymes in engineered *S. cerevisiae* to produce campesterol, Their above-noted paper sets out comparison of the ergosterol biosynthetic pathway in yeast, the phytosterol biosynthetic pathway in plants and engineered plant steroid pathways in yeast for example to synthesize β-sitosterol. 24-methylenecholesterol is thereby merely referred to as an engineered biosynthetic pathway intermediate and there is no suggestion to produce 24-methylenecholesterol. Whilst Xu et al. suggest that phytosterol synthesis established in *S. cerevisiae* should also be considered for reconstitution in *Y. lipolytica* for manufacturing purpose, they even hypothesize that 24-methylenecholesterol is toxic for yeast and may be a major cause of growth deficiency in *S. cerevisiae* strain YYL67 (*dwf1/5/7.MVA1,*Δare1/are2/erg4, *evolved*).

Yang et al (2021) Biomolecules, *Engineering of Saccharomyces cerevisiae for 24- Methylene-Cholesterol Production* nevertheless teaches that *S. cerevisiae* can be engineered to produce 24-methylenecholesterol by disrupting both the ERG4 and ERG5 genes and expressing a 7-dehydrocholesterol (DHCR7) enzyme. However, no suggestion is made for transfer of this approach to *Y. lipolytica*. Indeed, previous campesterol production in engineered *Y. lipolytica* is merely noted by Yang et al. as low yield and not satisfying large scale fermentation production. Rather they point to three approaches for addressing commercial production of 24-methylenecholesterol: searching for new potential microbial chassis strains, rational design of biosynthetic pathways and thirdly characterising further DHCR7 enzymes. The reported studies focus on testing the DHCR7 of *Physalis angulate* in *S. cerevisiae* with disrupted ERG4 and ERG5 genes for this purpose. However, on the basis of their studies the same authors merely go on to favour instead expression of duplicated codon-optimised sequences encoding *Xenopus laevis* DHCR7 and suggest still significant room for improvement of 24-methylenecholesterol yield.

Thus, a commercially viable method for producing 24-methylenecholesterol is still required either alone or as part of a sterol mixture.

The inventors in this instance set out to provide improved phytosterol production in yeast with one aim being to address this problem and provide engineered yeast synthesising 24-methylenecholesterol, either as a sole purified sterol, or as part of a mixture, e.g. including campesterol preferably as the 24α epimer, e.g. for use in preparing artificial bee nutrition, by firstly selecting an oleaginous yeast.

In so doing, it has been established that in such a yeast required disruption of ergosterol synthesis may not be compatible with adequate growth for useful heterologous gene expression, or indeed any colony growth, on a conventional yeast growth medium, e.g. at 30°C on yeast extract peptone

dextrose (YPD) medium containing 10g/l yeast extract, 20g/l peptone and 20g/l glucose, supplemented with 20g/l agar for preparation of solid medium. In other words, knock-out of any of the ERG4, ERG5 and ERG6 genes may simply lead to undesirable growth deficiency or no growth as illustrated by the studies reported herein in Example 2 on unsuccessful ERG4, ERG5 and ERG6 gene knock out in the *Y. lipolytica* strain ST9100. Undesirable growth deficiency in this context may be equated with a growth rate which is economically unviable on an industrial scale, such as a reduction in the maximum achievable biomass accumulation below 10g cell dry weight per litre of culture, e.g. when cells are grown at 30°C on yeast peptone dextrose (YPD) medium containing glucose as the carbon source.

This was an unexpected problem given the earlier studies noted above on campesterol production by different ERG5-knock out *Y. lipolytica* strains. Previously, ERG4 was identified as essential by transposon mutagenesis but only in the wild-type strain W29 (CLIB89/ ATCC20460™, Patterson et al. (2018) Metab. Eng. 48, 184-196). Gene essentiality has also been examined by CRISPR-Cas9 mediated gene disruption in *Y. lipolytica* PO1f (MatA, leu2-270, ura3- 302,xpr2-322, axp-2, Schwartz et al. (2019) Metab. Eng. 55, 102-110). Classification of ERG4 essentiality was inconclusive and varied according to the criterion used. However, the ST9100 strain is a modified strain for increased squalene synthesis, first reported in Arnesen et al. (2020) Front. Bioeng. Biotech. *Yarrowia lipolytica strains engineered for production of terpenoids.* It has been hypothesized by the inventors that inability to knock out ERG4, ERG5 or ERG6 in this strain without further engineering as taught herein may be related to the resulting accumulation of sterol intermediates which are toxic in high concentrations, for example due to detrimental effects to the membrane. Nevertheless, the *Y. lipolytica* strain ST9100 and other similar *Y. lipolytica* strains with increased squalene synthesis as a precursor to the sterol pathway are now enabled by teaching herein as highly desirable host cells for engineering to produce high levels of non-native sterols with appropriate gene knock-out.

As further discussed below, this gene knock out issue has been addressed by intracellular provision of a sterol surrogate as illustrated in the exemplification by introduction of a squalene-tetrahymanol cyclase coding sequence, e.g. *Tetrahymena thermophilia* squalene-tetrahymanol cyclase sequence (TtSTC coding sequence) under the control of a weak yeast promoter. Introduction of such a coding sequence into yeast has previously been suggested in Published International Application WO2021/133171 (Technische Universiteit Delft) but only in *Saccharomyces* species, e.g. *S. cerevisiae* and *Kluyveromyces* species, e.g. *Kluyveromyces marxiamus* to enable growth anaerobically without sterol supplements. However, WO2012/133171 does not address the problem of gene knock out addressed herein and need to balance expression of a sterol surrogate coding sequence such as a *TtSTC* coding sequence with required flux through a biosynthetic pathway for high production of a non-native sterol in an engineered *Y. lipolytica* strain.

The prior art does not provide for creating an industrially viable source of non-native sterol through a yeast with a high sterol content relative to wild type strains. In addition, the prior art does not address the issue of essentiality of certain genes involved in ergosterol production in any oleaginous yeast. Furthermore, the prior art fails to provide scalable methods for the production of a range of sterols at suitable titres for commercial use of such sterols.

As such, there is a need for improved methods of producing sterols. For example, methods that provide increased production of a range of sterols by engineered yeast. There is also a need for improved genetically modified yeast for use in such methods that have improved feasibility.

It is an aim of certain embodiments of the invention to provide yeast for the improved production of sterols and compounds derived therefrom, for example, with increased titre of sterols as well as a broader range of sterols than that produced in the prior art.

It is an aim of certain embodiments to provide improved methods for producing sterols.

It is an aim of certain embodiments to provide improved methods for producing and extracting sterols from the yeast for use in feeds and pharmaceuticals.

It is an aim of certain embodiments to provide products such as artificial dietary compositions, food products and pharmaceutical compositions that include sterols produced by the methods and yeast described herein.

It is an aim of certain embodiments to provide products such as artificial dietary compositions, food products and pharmaceutical compositions that include the yeast as described herein.

Summary of Invention

The invention provides genetically modified oleaginous yeast capable of producing a sterol surrogate such as tetrahymanol, such that normal ergosterol biosynthesis is no longer essential. As a result, certain genes involved in ergosterol biosynthesis are no longer essential and their expression can be partially or totally reduced. The sterol composition of such modified strains can be dramatically altered to achieve production of desired non-native sterols with high purity and content.

Thus provided in a first aspect of the invention is an oleaginous yeast for expression of one or more heterologous genes for production of one or more desired non-native sterols or compounds derived therefrom, wherein

(i) the yeast has reduced production of ergosterol compared with a wild-type oleaginous yeast or is incapable of producing ergosterol; and

(ii) wherein the yeast is provided with a sterol surrogate to aid cell growth.

A suitable reference wild-type yeast may be a corresponding wild type of the chosen host cells or conveniently for any oleaginous host cells, and especially such engineered *Y.lipolytica* cells, e.g. *Y.lipolytica* W29 strain Y-63746 as commercially available from the ATCC as ATCC20460™ or from the ARS culture collection, NCAUR, United States

As indicated above, such provision of a sterol surrogate will be desirable whenever the oleaginous yeast ahead of such engineering to modify sterol production is resistant to knock-out of any of the ERG4, ERG5 and ERG6 genes required for ergosterol synthesis via the native yeast ergosterol pathway (see Figure 2) such that such knock out results in undesirable growth deficiency or no growth. This may be associated with, for example, increased flux toward the sterol pathway such as increased squalene synthesis compared with wild-type as observed for *Y. lipolytica* ST9100. Thus, it may be associated with any of increased synthesis of squalene or another sterol precursor or sterol

pathway intermediate compared to a suitable reference strain. As indicated above, a growth rate is desired which is economically viable on an industrial scale. Hence undesirable growth deficiency may be equated with a reduction in the maximum achievable biomass accumulation below 10g cell dry weight per litre of culture, e.g. when employing conventional YPD medium containing glucose as the carbon source at 30°C.

Y. lipolytica ST9100 is an example of a preferred *Y. lipolytica* strain for engineering to provide a yeast in accordance with the invention, i.e. wherein a sterol surrogate is provided to compensate for any native gene for yeast ergosterol production which cannot otherwise be deleted without undesirable growth deficiency or no growth and permit expression of one or more required heterologous genes for desired alternative sterol production. This strain as indicated above is a prior described strain in Arnesen et al. (2020) Front. Bioeng. Biotech. It can be obtained starting from the commercially available *Y. lipolytica* W29 (MatA) strain Y-63746 as available from the ATCC as ATCC20460™ or from the ARS culture collection, NCAUR, in accordance with the strain development set forth by Arnesen et al., i.e. via initial production of the strain ST6512 strain as described in Marella et al. (2020) Metabolic Eng. 61,427-436 (also available from Euroscarf as accession no. Y41408). Details for such strain development are also provided in the exemplification herein. It will be appreciated that similar strains to ST9100 with increased squalene synthesis may be developed starting from *Y. lipolytica* Y-63746 and may be similarly employed as the platform strain for provision of a yeast in accordance with the invention suitable for producing one or more desired non-native sterols with simultaneous provision of a sterol surrogate.

It will be appreciated such sterol surrogate provision may be achieved by intracellular expression of a nucleic acid sequence encoding an enzyme for indirect or preferably direct provision of the sterol surrogate, e.g. such a coding sequence optimised for the particular yeast host. This may be especially preferred where the oleaginous yeast cells are incapable of taking up exogeneous sterols or sterol surrogates from the environment. Further, it will be recognised that the expression of the sterol surrogate will be controlled with a view to preventing diversion of carbon flux from impeding desired non-native sterol production, i.e. the engineered oleaginous yeast must remain suitable for production of the desired one or more non-native sterols, e, g. campesterol and/or 24-methylenecholesterol. Hence, for example, it may be found preferable for expression of the sterol surrogate to be under the control of a weak promoter in the chosen yeast cells. Such a promoter may, for example, be the PrGPAT promoter or PrDGA1 promoter as referred to in Holkenbrink et al.(2018) Biotech. J. EasyCloneYALI: CRISPR/Cas9-Based Synthetic Toolbox for Engineering of the yeast *Yarrowia lipolytica*, but may be any yeast promoter (synthetic or natural) which provides similar function as determined by conventional fluorescence assay of control of GFP expression. A suitable assay for this purpose is also described in the above-noted paper of Holkenbrink et al. It will be appreciated, however, that other known means for achieving low level expression may alternatively be employed, e.g. promoter truncation, feedback regulation etc.

Preferably, the sterol surrogate may be tetrahymanol. This may be especially preferred where the oleaginous yeast cells, e.g. *Y. lipolytica* cells, have increased squalene synthesis compared with wild type. Thus the oleaginous yeast may comprise a heterologous nucleic acid encoding a squalene-

tetrahymanol cyclase, preferably such a coding sequence optimised for expression in the yeast. For example, the squalene-tetrahymanol cyclase may be the *Tetrahymanol thermophilia* squalene-tetrahymanol cyclase or a functional variant thereof for providing the sterol surrogate.

It will be appreciated however that an alternative sterol surrogate may be provided. For example, the sterol surrogate may be a hopanoid. Again, this may be especially preferred where the oleaginous yeast cells, e.g. *Y. lipolytica* cells, have increased squalene synthesis compared to wild type. Thus, the oleaginous yeast may comprise a heterologous nucleic acid encoding a squalene-hopene cyclase, preferably codon-optimised for expression in the yeast. For example, the squalene-hopene cyclase may be a *Schizosaccharomyces japonicus* squalene-hopene cyclase or a functional variant thereof for providing the sterol surrogate.

As indicated above, the sole purpose of the sterol surrogate is to permit deletion of one or more genes for ergosterol synthesis which otherwise is precluded by growth deficiency or no growth, i.e. compensate for essentiality of a gene, e.g. ERG4 where production of 24-methylenecholesterol is desired or ERG6 where production of cholesterol is desired. It will be understood therefore that as described herein, the term "sterol surrogate" relates to a non-native sterol or functionally equivalent triterpenoid but as implied by the term "surrogate" has a distinct purpose which is distinct from the one or more non-native sterols which it is desired to produce for further application. For example, non-native sterol refers to any plant phytosterol such as desmosterol, cholesterol, 24-methylenecholesterol, campesterol, isofucosterol, beta-sitosterol or stigmasterol which may be desired alone or as part of a mixture of such sterols.

It will also be understood that the yeast provided herein may provide a commercial or industrially relevant amount of non-native sterol as described herein. For example, as further illustrated by the exemplification, a *Y. lipolytica* yeast in accordance with the invention, derived from *Y. lipolytica* strain ST9100 by ERG5 knock out and intracellular expression of a heterologous delta-7 sterol reductase, can enable a campesterol yield of 40-42 mg/g dry cell weight (DCW). As also illustrated herein, a *Y. lipolytica* strain in accordance with the invention derived from *Y.lipolytica* strain ST9100 with both the native ERG4 and ERG5 genes knocked out and expressing a heterologous delta-7 sterol reductase can enable a 24-methylenecholesterol yield of more than 40 mg/g DCW, e.g. about 48 mg/g DCW. As additionally illustrated by the exemplification, *a Y. lipolytica* yeast in accordance with the invention and derived from *Y. lipolytica* strain ST9100 to express intracellularly a sterol surrogate can provide mixtures of non-native sterols of industrial use wherein the yield of 24-methylenecholesterol is still industrially significant, e.g. as much as 9-10 mg/g DCW, for example, in a mixture also including campesterol and cholesterol. Indeed, engineered *Y. lipolytica* strains have been achieved which provide still higher 24-methylenecholesterol production, above 15mg/g DCW e.g. about 18-23 mg/g DCW, e.g. between about 22-23 mg/g DCW, as the dominant non-native sterol in a non-native sterol mixture including measurable campesterol and importantly campesterol as the plant (24R) epimer, e.g. a sterol mixture providing 24-methylenecholesterol together with all of campesterol, cholesterol, isofucosterol, desmosterol in quantifiable amount and detectable β-sitosterol as illustrated herein by genetically engineered *Y. lipolytica* strain ST12178. Similar non-native sterol production might be expected with other oleaginous yeast engineered in accordance with the invention which also exhibit

increased squalene synthesis, especially other such engineered *Y. lipolytica* strains. Such production of 24-methylenecholesterol, either alone or in a mixture, may be especially useful for example for provision of phytosterols in a food composition where a domesticated insect like the honeybee requires several sterols in its diet.

Optionally, the oleaginous yeast of the first aspect comprises an attenuated or deleted endogenous sterol C-22 desaturase (ERG5). Optionally, the oleaginous yeast further comprises an attenuated or deleted endogenous delta-24 sterol reductase (ERG4). Optionally, the oleaginous yeast comprises an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6). Optionally, the oleaginous yeast comprises an attenuated or deleted endogenous sterol C-22 desaturase (ERG5) and further comprises an attenuated or deleted endogenous delta-24 sterol reductase (ERG4), and/or sterol C-24 methyltransferase (ERG6).

Preferably, the oleaginous yeast of the first aspect is further engineered to comprise one or more heterologous nucleic acid sequences capable of expression to provide one or more of:

a. a delta-7 sterol reductase enzyme;
b. a delta-24(28) sterol reductase enzyme;
c. a delta-24(25) sterol reductase enzyme;
d. a sterol C-28 sterol methyltransferase enzyme and
e. a sterol C-22 desaturase enzyme

whereby production of the desired one or more non-native sterols or compounds derived therefrom can be achieved. One or more heterologous delta-24(28) sterol reductase and/or delta-24(25) sterol reductase may be provided as further discussed below depending on the non-native sterol or sterols required.

Thus, an oleaginous yeast of the invention expressing a sterol surrogate, e.g. tetrahymanol, may have any combination of (i) endogenous gene deletion or attenuation(s) with (ii) expressible heterologous gene(s) as set out in Table 2 with a view to producing a specific sterol, e.g. campesterol or 24-meththylenecholesterol or a mixed sterol composition.

More specifically, such a yeast may be an oleaginous yeast as follows:

a. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising campesterol (see Figure 4);

b. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising 24-methylenecholesterol (see Figure 5);

c. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6), optionally an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or optionally an attenuated or deleted delta-24 sterol reductase enzyme (ERG4) and;
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising desmosterol (see Figure 6);

d. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted sterol C-24 methyltransferase (ERG6);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme; and
(iii) a heterologous nucleic acid sequence encoding a delta-24 sterol reductase enzyme,
whereby one or more non-native sterols are produced comprising cholesterol (see Figure 7);

e. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and
(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme,
whereby one or more non-native sterols can be produced comprising isofucosterol (delta-24(28)-Z isomer) or fucosterol (delta-24(28)-E isomer) (see Figure 8 and Figure 16b);

f. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and
(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme,
wherein one or more non-native sterols can be produced comprising beta-sitosterol (see Figure 9)

g. wherein the oleaginous yeast further comprises:
(i) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and
(ii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme
whereby one or more non-native sterols are produced comprising stigmasterol;
optionally,

wherein the oleaginous yeast has an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or delta-24 sterol reductase enzyme (ERG4) and optionally additionally one or more further heterologous nucleic acid sequences are provided to express a DWF1 enzyme and /or sterol C-22 desaturase enzyme (see Figure 10)

h. wherein the oleaginous yeast further comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated delta-24 sterol reductase enzyme (ERG4), preferably where the ERG5 gene is deleted and the activity of ERG4 is attenuated by provision of the same encoding sequence, or a corresponding plant delta-24(28) sterol reductase (DWF1) coding sequence, under the control of a weak promoter selected from PrDGA1 and functionally equivalent weak yeast promoters;
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, optionally a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase and/or optionally a heterologous nucleic acid sequence encoding a C-28 sterol methyl transferase, whereby a non-native sterol mixture can be produced, preferably such that a sterol mixture can be produced comprising both 24-methylenecholesterol and campesterol, optionally together with one or more further non-native sterols in detectable amount, e.g. cholesterol.

Where it desired to produce a sterol mixture as in (h) above in which campesterol is present as the plant (24R) epimer, it will be recognised that normal yeast delta-24 sterol reductase activity will in effect be attenuated with additional consequential change of stereochemistry to ensure production of campesterol as the 24R epimer by replacement of the ERG4 gene by a coding sequence for an enzyme functioning as a plant delta-24(28) reductase (i.e.DWF1 enzyme) under the control of a weak yeast promoter as defined. "Plant DWF1 coding sequence" in this context will be understood as a coding sequence providing any enzyme with the characteristics of a plant delta-24(28) reductase capable of converting some 24-methylenecholesteol to campesterol as the 24R epimer. Conveniently, the DWF1 encoded may be one of many naturally occurring such enzymes, e.g. the delta- 24(28) of *Solanum tuberosum.* Where production of the plant 24R epimer of campesterol is not an overriding consideration, then attenuation of yeast ERG4 may be by simply providing an alternative to the native ERG4 gene in which the native ERG4 coding sequence is under the control of a weak yeast promoter.

Both *Solanum tuberosum* and *Solanum lycopersicum* possess two delta-24 sterol reductase variants, one catalyses delta-24(25) reduction and the other catalyses delta-24(28) reduction. While such a delta-24(28) sterol reductase can be employed as a substitute for yeast ERG4 activity, it will be recognised that for cholesterol production as descried above a heterologous coding sequence will be provided encoding a delta-24(25) sterol reductase. Preferably for example the delta-24(25) sterol reductase of *Solanum lycopersicum* may be chosen for this purpose as illustrated by the exemplification provided below.

Where a heterologous coding sequence is referred to above it will be appreciated that generally this will be a coding sequence codon-optimised for the yeast host species. Heterologous nucleic acid sequences may be provided, for example, encoding all of a delta-7 sterol reductase with deletion of the ERG5 gene, a plant DWF1 as discussed above to in effect substitute for the native ERG4 gene and additionally both a delta-24(25) sterol reductase and a C-28 sterol methyltransferase whereby a sterol mixture can be obtained comprising 24-methylenecholesterol, the plant (24R) epimer of campesterol and measurable cholesterol together with other non-native sterols. 24-methylenecholesterol may preferably be the dominant sterol in such sterol mixture which may contain additional non-native sterols in at least detectable amount, e.g. all of isofucosterol, desmosterol and beta-sitosterol.

As indicated above as an alternative to PrDGA1 as a weak yeast promoter may be chosen PrGPAT as disclosed in Holkenbrink et al (2018) *ibid*. However, as also indicated above functionally equivalent weak yeast promoters may be readily determined in well-known manner. Any promoter may be employed that gives expression in the same genomic context capable of fulfilling the same purpose. Such determination may for example include conventional fluorescence assay of control of GFP expression in appropriate yeast host cells, e.g. *Y. lipolytica* yeast cells. The term 'weak yeast promoter' as used herein will be understood to include promoters that by such assay in *Y. lipolytica* yeast cells, e.g. the reference wild-type *Y.lipolytica* W29 strain Y-63746, show lower activity than the well-known PrTEFintron promoter as first employed for oil production in *Y. lipolytica* (Tai and Stephanopoulos, Metab. Eng. (2013) 1-9). For the sequence of the PrTEFintron reference may again be made to the supplementary information of the Holkenbrink et al (2018) paper. The term 'PrGPAT promoter' as used herein will be understood to always equate with the sequence given for that promoter in the referenced 2018 paper of Holkenbrink et al. (i.e. the sequence corresponding to the promoter region of gene YALI1_C00209g in *Y. lipolytica* W29 strain Y-63746, with genomic location of the promoter being Chromosome 1C:20927-22056, and genomic location of the downstream gene being Chromosome 1C: 18,422-20,926)

With regard to a yeast of the invention as above for production of stigmasterol, although no endogenous genes are deleted in this case, an optimal activity of C-28 sterol methyltransferase can be expected to divert much, if not all, pathway flux away from ergosterol biosynthesis towards stigmasterol production. In this case, ergosterol would be absent or too low to fulfil cellular sterol requirements such as membrane requirements. Stigmasterol may not adequately substitute for ergosterol. Presence of tetrahymanol or another sterol surrogate would therefore be beneficial to such a strain in order to fulfil the membrane requirements and improve cell fitness thereby aiding growth.

In some instances of engineered yeast strains of the invention, it may prove beneficial to employ more than one heterologous coding sequence to provide a required activity, for example where C-28 sterol methyltransferase activity is required one or more C-28 sterol methyltransferase coding sequences may employed. Where more than one heterologous coding sequence is provided for a required enzyme activity such coding sequences may encode the same enzyme or different enzymes, e.g. 2 or 3 different enzymes. For example, beta-sitosterol and isofucosterol-producing engineered *Y. lipolytica* strains may be provided as described herein with up to 3 different C-28 sterol

methyltransferase genes. Increasing the copy number of heterologous genes (same or different variants) for any required activity may be advantageous to increase pathway flux to a desired product.

Just as campesterol may be produced as one of two epimers in a yeast of the invention, the same applies for isofucosterol. It is recognised that isofucosterol can be produced, for example, in green plants as the delta-24(28)-Z isomer or can be found alternatively as the delta-24(28)-E isomer, for example in red algae and as such is commonly referred to as fucosterol. This arises from the nature of the C-28 methyltransferase (SMT; EC2.1.1.143) responsible as shown in Figure 16b. Depending on the C-28 sterol methyltransferase provided for isofucosterol production in a yeast of the invention, either the Z isomer or E isomer may thus be attained. Hereinafter the term 'isofucosterol 'should be taken to embrace production of either isomer, unless a specific plant C-28 sterol methyltransferase is specified as in the exemplification which ensures the Z-isomer.

The oleaginous yeast may be any oleaginous yeast where provision of the sterol surrogate is required to facilitate cell growth in the face of the specified gene alterations including any deletion or deletions in the ergosterol biosynthesis pathway. Preferably for example, the oleaginous yeast may be an engineered *Y.lipolytica* production strain, for example such a strain engineered from *Y. lipolytica* ST9100 or another *Y. lipolytica* which shares all or some of the same modified genotype features of *Y. lipolytica* ST9100 compared to *Y. lipolytica* W29 strain Y-63746 as the reference strain with increased synthesis of squalene, or possibly another sterol precursor or sterol pathway intermediate, compared to that reference strain.

It will be understood that "deleted" equates with non-functional and thus alternatively a deleted gene may be described as knocked-out. This may be achieved in any known manner, e.g. using a CRISPR-Cas/gRNA or marker-mediated gene deletion.

As regards (a) above, it will be recognised that preferably the endogenous ERG4 gene may be additionally substituted by a plant delta-24(28) sterol reductase (DWF1; e.g. the DWF1 of *Arabidopsis thaliana* or more preferably the delta-24(28) sterol reductase of *Solanum tuberosum*) such that the 24α configuration (R) of campesterol is attained. Again it will be recognised that the designation 'plant delta-24(28) sterol reductase' can be applied to any delta-24 reductase which ensures the correct plant stereochemistry at the C24 position when converting 24-methylenecholesterol to campesterol as shown in Figure 16 . As indicated above, this may be highly preferred for downstream uses of campesterol, e.g. for provision in honeybee feed. For example, the moulting hormone Makisterone A is produced from campesterol in bees. Provision of the 24β epimer of campesterol would lead to production of epi-Makisterone A, which may not function in the same way. The same ERG4 gene substitution may be additionally favoured in producing beta-sitosterol and stigmasterol; see Figures 9 and 10. As already discussed above, it may also be favoured in producing a sterol mixture in accordance with (h) above comprising 24-methylenecholesterol and campesterol, e.g. particularly such a sterol mixture to be used as a source of sterols in an artificial feed for honeybees or other insects or animals.

Plant DWF1 enzymes that may be employed for substitution of ERG4 as above include by way of example any of *Solanum tuberosum* delta-24(28) sterol reductase (NCBI seq. ref: BAQ55274.1),

Arabidopsis thaliana delta-24 sterol reductase (NP_850616.1), Arachis duranensis delta-24 sterol reductase (XP_015952627.2), *Selaginella moellendorffii* delta-24 sterol reductase (XP_002960921.1), *Capsicum chinense* delta-24 sterol reductase (PHU28681.1), *Artemisia annua* delta-24 sterol reductase (PWA66182.1), *Helianthus annuus* delta-24 sterol reductase (XP_022012299.1), *Cocos nucifera* delta-24 sterol reductase (EHA8587492.1), *Triticum urartu* delta-24 sterol reductase (EMS57493.1), *Gracilariopsis chorda* delta-24 sterol reductase (PXF44537.1), *Capsella rubella* delta-24 sterol reductase (XP_006297345.1) and *Ajuga reptans var. atropurpurea* delta- 24 sterol reductase (BAS68578.1)

Yeast in accordance with the invention as discussed above which are provided with a sterol surrogate may be specifically engineered to produce sterol mixtures of interest with provision of heterologous genes in combination with endogenous ERG5 gene deletion or attenuation, preferably deletion, and attenuation of ERG4 activity, for example preferably whereby 24-methylenecholesterol is produced in combination with campesterol, possibly with one or more further phytosterols, e.g. cholesterol. Preferably, 24-methylenecholesterol may be the major sterol of the mixture, e.g. at a level of about 2-3 mg/g or higher DCW, possibly as high as 9-10 mg/g DCW or as indicated above even considerably higher, e.g. at least 15mg/g DCW, preferably at least about 18 or even at least 20 mg/g DCW. In this case, the endogenous ERG5 gene may be deleted in conventional manner, e.g. using CRISPR-Cas9/gRNA. Attenuation of endogenous ERG4 activity may be achieved by changing the promoter of the native ERG4 gene to a weaker promoter e.g. deleting the native ERG4 gene and re-introducing the ERG4 coding sequence under the control of a weaker yeast promoter, e.g. a weak yeast promoter as discussed above such as a PrDGA1 promoter. Alternatively, the endogenous ERG4 gene may be substituted by a plant DWF1 coding sequence under the control of such a promoter. Indeed, as noted above, this may be favoured so that the plant-produced epimer of campesterol is attained in the resulting non-native sterol mixture. As indicated above, this is combined with expression of a heterologous delta-7 sterol reductase, optionally with a delta- 24(25) sterol reductase or C-28 sterol methyltransferase or both a delta- 24(25) sterol reductase and a C-28 sterol methyltransferase. Expression of a heterologous delta-7 sterol reductase with just a delta-24(25) sterol reductase may be preferred. This is so since the delta-24(25) sterol reductase will permit conversion of 24-methylenecholesterol to cholesterol. Expression may be controlled so that all of 24-methylenecholesterol, campesterol and cholesterol is attained at measurable amount. See Example 4. In this way, a *Y. lipolytica* derived from strain ST9100 has been attained which produces nearly 10 mg/g DCW of 24-methylenecholesterol, together with both significant campesterol and cholesterol and unexpectedly some desmosterol.

It will be appreciated that such an advantageous sterol mixture in which 24-methylenecholesterol is combined with campesterol and cholesterol, preferably with yeast expression of a DWF1 sequence to attain the correct plant epimer of campesterol, may also be attained by equivalent engineering of alternative oleaginous yeast strains, e.g. *Y. lipolytica* strains which like ST9100 have increased squalene synthesis compared with the corresponding wild type.

It will be recognised that advantageous sterol mixtures which can be attained by engineering of yeast strains of the invention also include such sterol mixtures in which 24-methylenecholesterol is

combined with isofucosterol, preferably such strains with deletion of ERG4 and ERG5 and in which expression of a SMT variant (i.e. C-28 sterol methyltransferase variant) ensures production of isofucosterol as the delta-24(28) Z-isomer. Suitable such C-28 methyltransferase variants include, for example, any of the C-28 methyltransferase variants of *Chenopodium quinoa*, *Arabidopsis thaliana* and *Amborella trichopoa*. Expression of one or more heterologous coding sequences for a C-28 methyltransferase will be combined with expression of one or more heterologous coding sequences for a delta-7 sterol reductase enzyme. Such production of isofucosterol is illustrated in the exemplification by engineering of *Y. lipolytica* strains derived from ST9100. Equivalent engineering of alternative oleaginous yeast strains, e.g. *Y. lipolytica* strains which like ST9100 have increased squalene synthesis compared with the corresponding wild type, is however again contemplated.

It will be appreciated that such an advantageous sterol mixture in which isofucosterol as the delta-24(28) Z-isomer is combined with 24-methylenecholesterol may additionally include cholesterol arising from additional expression of a delta-24(25) sterol reductase. Again such sterol mixture production may be attained by equivalent engineering of various alternative oleaginous yeast strains, e.g. *Y. lipolytica* ST9100 and other *Y. lipolytica* strains which like ST9100 have increased squalene synthesis compared with the corresponding wild type.

As indicated above, neither 24-methylenecholesterol nor isofucosterol has previously been available industrially. Significantly, by means of yeast of the invention isofuctosterol can be obtained, preferably as the delta-24(28)-Z isomer, for incorporation into compositions and hence artificial dietary compositions for bees and other insects or animals can now be disclosed for the first time which incorporate this sterol. It will be appreciated that such incorporation may be preferably as part of a sterol mixture attained in accordance with the invention additionally comprising 24-methylenecholesterol, for example comprising both 24-methylenecholesterol and campesterol as the 24R plant epimer.

As now further highlighted, especially preferred may be expression of a heterologous delta-7 sterol reductase with a delta-24(28) sterol reductase (DWF1), a delta-24(25) sterol reductase and C-28 sterol methyltransferase accompanied by deletion of the endogenous ERG5 gene and attenuation of ERG4 activity to again attain a sterol mixture comprising all of 24-methylenecholesterol, campesterol, preferably as the plant epimer in view of expression of a plant DWF1 coding sequence in substitution for yeast ERG4, and cholesterol plus additional sterols in detectable amount. In this way, a *Y. lipolytica* derived from strain ST9100, or a similar *Y. lipolytica* strain as discussed above, may be attained capable of producing 24-methylenecholesterol as the dominant sterol plus other sterols including all of campesterol as the plant (24R) epimer, cholesterol, isofucosterol and desmosterol and possibly also beta-sitosterol in at least detectable amount, for example where the production of 24-methylenecholesterol is at least 20 mg/g DCW, e.g. about 22-23 mg/g DCW, but other sterols are also attained at measurable amount, including all of campesterol, cholesterol, isofucosterol and desmosterol. It has been shown that such a sterol mixture may be achieved in which campesterol as the plant epimer is present in an amount of at least 2-3 mg/g DCW and cholesterol is also present at higher amount, e.g. at least 8-9 mg/g DCW, together with isofucosterol and desmosterol. Such a strain is illustrated by *Y. lipolytica* strain ST12178, the engineering of which is described in Example 4.

Production of a sterol mixture by an oleaginous yeast of the invention in which 24-methylenecholesterol is present with at least campesterol as the plant epimer but preferably also all of at least cholesterol, isofucosterol and desmosterol, preferably additionally with beta-sitosterol, is especially of interest in relation to provision of a sterol mixture in a dietary composition for honeybees. For this purpose, sterols may be isolated from yeast cells of the invention. However, alternatively yeast cells of the invention may be recovered from culture as a yeast biomass which is then inactivated, e.g. heat-inactivated, and dried, e.g. by heating at no more than 60°C. The dried yeast biomass may then be preferably converted to a powder. Such a dried yeast biomass can be stored frozen, e.g. at -20°C, prior to use, e.g. incorporation into a composition such as a dietary composition. While such a dietary composition is of especial interest to the inventors for provision as a feed to honeybees, it will be appreciated that such compositions may have wider use, e.g. in the healthcare field for humans and animals. The sterol composition could be adjusted as required in each case. In some instances, a non-yeast produced sterol supplement may be added, e.g. such a supplement comprising beta-sitosterol.

In the case of oleaginous yeast strains of the invention expressing both a delta-24(25) sterol reductase and a C-28 sterol methyltransferase so as to produce a mixture of phytosterols, it has additionally surprisingly been found that omission of the sterol surrogate gene from such a sterol mixture producing strain may be carried out with retention of useful phytosterol mixture production including campesterol, 24-methylenecholesterol and cholesterol, making up the majority of the mixture. Again by using a DWF1 plant enzyme coding sequence under the control of a weak promoter, campesterol may be attained in industrially significant amount with the plant stereochemistry at the C-24. The delta-24(25) sterol reductase and C-28 sterol methyltransferase may also be expressed employing a weak yeast promoter such as a PrGPAT promoter as disclosed in Holkenbrink et al (2018) *ibid*.

The studies reported herein enable other genetic modifications of oleaginous yeasts taught above to be extended for the first time to such yeast without a sterol surrogate gene to attain desired non-native sterol or non-native sterol mixture production. The sterol composition of such modified strains may nevertheless be dramatically altered to achieve commercially useful production of exogenous sterols with high purity and titre.

In relation to such embodiments of the invention as now discussed below, it is not deemed feasible for any sterol pathway engineering in a facultative anaerobic yeast such as *S. cerevisiae* to be assumed to be immediately transferable to an obligate aerobic yeast such as *Y. lipolytica*. In this connection, it is for example, especially noteworthy that ERG6 has been found to be essential in *Y. lipolytica* ST9100, as will be returned to below in relation to cholesterol production, as well as in the wild-type *Y. lipolytica* strains W29 (CLIB89/ ATCC20460™, Patterson et al. (2018) Metab. Eng. 48, 184-196) and PO1f (MatA, leu2-270, ura3- 302,xpr2-322, axp-2, Schwartz et al. (2019) Metab. Eng. 55, 102-110).

Thus in a further aspect of the invention, there is provided an oleaginous yeast for production of at least one non-native sterol, wherein the at least one native sterol comprises 24-methylenecholesterol or a derivative thereof, the yeast comprising:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4); and

(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme.

In another aspect, there is provided an oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises desmosterol or a derivative thereof, the yeast comprising:

(i) an attenuated or deleted endogenous sterol C-24 methyl-transferase (ERG6), optionally an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or optionally an attenuated or deleted delta-24 sterol reductase enzyme (ERG4) and;

(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme.

In a still further aspect, there is provided an oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises isofucosterol (delta-24(28)-Z isomer) and/or fucosterol (delta-24(28)-E isomer) or a derivative thereof, the yeast comprising:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);

(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme; and

(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyl-transferase enzyme.

In another aspect, there is provided an oleaginous yeast for production of at least one non-native sterol, wherein the at least one native sterol comprises cholesterol or a derivative thereof the oleaginous yeast comprising:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted sterol C-24 methyl-transferase (ERG6);

(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme;

(iii) a heterologous nucleic acid sequence encoding a delta-24 sterol reductase enzyme.

As noted above, ERG6 is essential in a *Y. lipolytica* such as *Y. lipolytica* ST9100 so a cholesterol-producing engineered such strain could not be produced by the same methods described to create cholesterol-producing strains of *S. cerevisiae* or *P. pastoris*. In contrast, a cholesterol-producing oleaginous yeast of the invention expressing a sterol surrogate such as tetrahymanol might be evolved to remove sterol surrogate dependency. The same extrapolation can reasonably be made for any sterol surrogate dependent yeast of the invention described above.

In another aspect, there is provided an oleaginous yeast for production of at least one non-native sterol, wherein the least one non-native sterol comprises beta-sitosterol or a derivative thereof, the oleaginous yeast comprising:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);

(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and

(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme.

In a still further aspect, there is provided an oleaginous yeast for production of at least one non-native sterol where the at least one non-native sterol comprises stigmasterol or a derivative thereof, the yeast comprising:

(i) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. a plant DWF5 and

(ii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme, e.g. a plant SMT2 ; optionally,

wherein the oleaginous yeast has an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or delta-24 sterol reductase enzyme (ERG4) and optionally additionally one or more further heterologous nucleic acid sequences are provided to express a plant delta-24(28) sterol reductase (DWF1) enzyme and /or sterol C-22 desaturase enzyme.

When the yeast includes an attenuated or deleted endogenous ERG5, the yeast will include a heterologous nucleic acid encoding a sterol C-22 desaturase enzyme, e.g. plant CYP710A. The yeast may include an attenuated or deleted endogenous ERG4 and have in substitution a heterologous nucleic acid sequence encoding a plant delta-24(28) sterol reductase enzyme (DWF1). This provides a yeast that produces stigmasterol in the same configuration (epimer) as found in plants. See Figure 10.

In yet another aspect, there is provided an oleaginous yeast for production of a non-native sterol mixture, the yeast comprising:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated delta-24 sterol reductase enzyme (ERG4), preferably where the ERG5 gene is deleted and the activity of ERG4 is attenuated by provision of the same encoding sequence, or a corresponding plant delta-24(28) sterol reductase (DWF1) coding sequence, under the control of a weak promoter selected from PrDGA1 and functionally equivalent weak yeast promoters;

(ii) a heterologous nucleic acid encoding a delta-7 sterol reductase enzyme, optionally a heterologous nucleic acid encoding a delta-24(25) sterol reductase and/or optionally additionally a heterologous nucleic acid encoding a C-28 sterol methyl transferase, whereby a non-native sterol mixture is produced, preferably such that a sterol mixture is produced comprising both 24-methylenecholesterol and campesterol, optionally together with one or more further non-native sterols, e.g. cholesterol.

Preferably, the oleaginous yeast may be the oleaginous yeast *Yarrowia lipolytica*, for example a yeast derived from *Yarrowia lipolytica* W29 strain Y-63746. As indicated above, especially preferred is a yeast derived from the *Y. lipolytica* strain ST9100 in which case a sterol surrogate may be provided intracellularly to enable required native gene deletion or attenuation but then removed from the strain after further engineering to provide heterologous coding sequences. As indicated above, this may for example be achieved and preferred where the required heterologous coding sequences enable a mixture of non-native sterols to be produced, e.g. all of campesterol, 24- methylenecholesterol,

cholesterol and desmosterol, possibly supplemented by isofucosterol and/or beta sitosterol, as exemplified herein.

Genetically modified yeast of the invention may be cultured in conventional manner for production of the one or more desired sterols or derivatives thereof, preferably with a simple carbon source such as glucose. The one or more sterols thus synthesized may be converted to one or more further sterol derivatives in the same cells or once isolated. The one or more sterols or derivatives thus produced may be further incorporated into a composition for dietary, cosmetic or pharmaceutical use (e.g. any of an artificial dietary composition for animals or insects such as bees, a food product, an agricultural composition, a cosmetic composition and pharmaceutical composition). As indicated above, yeast of the invention may for example be genetically-modified with a view to specifically addressing the desire for sterols for incorporation into artificial bee feed, for example, production from a single genetically-modified yeast strain of a mixed sterol composition comprising 24-methylenecholesterol and campesterol, possibly with one or more other phytosterols. It may be chosen to simply incorporate yeast cells of the invention into an artificial dietary composition, e.g. for bees, without a sterol extraction step. In this case as discussed above such yeast cell incorporation may be in the form of a dried yeast cell biomass attained following recovery of yeast cells from a culture medium, e.g. culture medium following growth of yeast cells by conventional fed-batch fermentation.

Embodiments of the invention are further described herein with reference to the accompanying drawings as set out below.

Brief Description of Figures

Figure 1 shows the chemical formula and nomenclature used for various sterols including, desmosterol, cholesterol, 24-methylenecholestreol, campesterol, isofucosterol, beta-sitosterol, and stigmasterol.

Figure 2 shows a schematic of the later stage of the biosynthetic pathway for sterol production in yeast.

Figure 3 shows a schematic of the production of a sterol surrogate in yeast. TtSTC1 (squalene-tetrahymanol cyclase) is introduced.

Figure 4 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce campesterol. The gene ERG5 (sterol C-22 desaturase enzyme) has reduced activity (for example is knocked out (KO)) and a heterologous DWF5 (delta-7 sterol reductase enzyme) is introduced.

Figure 5 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce 24-methylenecholesterol. The genes ERG5 (sterol C-22 desaturase enzyme) and ERG4 (delta-24 sterol reductase enzyme) have reduced activity (for example are knocked out (KO)) and heterologous DWF5 (delta-7 sterol reductase enzyme) is introduced.

Figure 6 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce desmosterol. The gene ERG6 (sterol C-24 methyl-

transferase) has reduced activity (for example is knocked out (KO)) and heterologous DHCR7 (delta-7 sterol reductase enzyme) is introduced. Optionally, the genes ERG5 (sterol C-22 desaturase enzyme) and ERG4 (delta-24 sterol reductase enzyme) also have reduced activity (for example are knocked out (KO)).

Figure 7 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce cholesterol. The genes ERG6 (sterol C-24 methyl-transferase) and ERG5 (sterol C-22 desaturase enzyme) have reduced activity (for example are knocked out (KO)) and heterologous DHCR7 (delta-7 sterol reductase enzyme) and DHCR24 (delta-24 sterol reductase enzyme) are introduced.

Figure 8 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce isofucosterol. The genes ERG5 (sterol C-22 desaturase enzyme) and ERG4 (delta-24 sterol reductase enzyme) have reduced activity (for example are knocked out (KO)) and heterologous DWF5 (delta-7 sterol reductase enzyme) and heterologous SMT2 (sterol C-28 methyltransferase enzyme) are introduced.

Figure 9 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce beta-sitosterol. The gene ERG5 (sterol C-22 desaturase enzyme) has reduced activity (for example is knocked out (KO)) and heterologous DWF5 (delta-7 sterol reductase enzyme) and heterologous SMT2 (sterol C-28 methyl-transferase enzyme) are introduced.

Figure 10 shows a schematic of genetic modifications made to the later stage of the biosynthetic pathway for sterol production in yeast to produce stigmasterol. Heterologous DWF5 (delta-7 sterol reductase enzyme) and heterologous SMT2 (sterol C-28 methyltransferase enzyme) are introduced. Optionally the gene ERG5 (sterol C-22 desaturase enzyme) has reduced activity (for example is knocked out (KO)) and CYP710A1 (sterol C-22 desaturase enzyme) is introduced.

Figures 11 A and B: **A**. compares sterol and tetrahymanol production by *Y. lipolytica* strains as referred to in Example 3. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation. **B**. compares sterol and tetrahymanol production in engineered *Y. lipolytica* strains according to the invention for the production of campesterol and 24-methylenecholesterol (ST11071 and ST11064) and in parental strains used in their construction (ST6512, ST9100 and ST1105, ST11027 an ST11040). STC= expressing a squalene- tetrahymanol cyclase. Deletion of the ERG4 and ERG5 genes is indicated by – erg4 and – erg5 respectively. DHCR7 = a delta-7 sterol reductase enzyme. Values again represent the mean of three technical replicates. Error bars represent ± 1 standard deviation.

Figure 12 shows campesterol production by strains ST11066 to ST11075. A different delta-7 sterol reductase source was used for each strain. Codon optimised coding sequences for each such variant enzyme were employed under the control of the PrTEFintron promoter. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation.

Figure 13 shows campesterol production by strains expressing *Ectocarpus siliculosus* delta-7 sterol reductase under the control of the PrTEFintron promoter in different genetic backgrounds. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation.

Figure 14 shows 24-methylenecholesterol production by strains ST11056 to ST11065. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation.

Figure 15 shows sterol and tetrahymanol production by various engineered *Y. lipolytica* strains as referred to in the exemplification. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation. Asterix (*) represents trace amount where value is below limit of detection for quantification.

Figure 16A shows a schematic of comparison of action of ERG4 (i.e. yeast sterol C-24 reductase) and DWF1 (i.e. plant delta-24(28) sterol reductase) leading to different stereochemistry at the C-24 position of a sterol such as campesterol.

Figure16B shows a schematic comparison of production of isofucosterol (delta-24(28)-Z isomer) or fucosterol (delta-24(28)-E isomer) relying on C-28 methyltransferases of different source.

Figure 17A shows (i) production of β-sitosterol, campesterol and tetrahymanol by engineered *Y. lipolytica* strains ST11804 and ST12139 as described in Example 4 and (ii) production of isofucosterol, 24-methylenecholesterol and tetrahymanol by engineered *Y. lipolytica* strains ST11803 and ST21208 as also described in Example 4. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation.

Figure 17B shows (i) desmosterol and tetrahymanol production by engineered *Y. lipolytica* strain ST11346 a described in Example 4 and (ii) production of cholesterol and tetrahymanol by engineered *Y. lipolytica* strains ST11829 and ST11830 as additionally described in Example 4. The only difference between strains ST11829 and ST11830 is the heterologous delta-24 sterol reductase provided in the cells via expression of a coding sequence codon-optimised *for Y. lipolytica* , the delta-24 sterol reductase from *Danio rerio* or the delta-24 sterol reductase from *Mus musculus* respectively. Strain ST11829 expressing the *Danio rerio* delta- 24 sterol reductase produced far higher cholesterol, almost twice as much at 26.6 mg/g DCW. Values represent the mean of three technical replicates. Error bars represent ± 1 standard deviation.

Figure 18A shows production of 24-methylenecholesterol and tetrahymanol in the ΔERG4ΔERG5 Y. lipolytica strain ST11064 expressing the delta-7 sterol reductase of *Tetraselmis* sp. GSL018 under the control of the PrTEFintron promoter and non- native sterol production of strains further engineered therefrom: (i) strain ST11943 additionally expressing the delta- 24(28) sterol reductase of *Solanum tuberosum* under the control of a weaker promoter to enable production of campesterol as the plant epimer and (ii) strain ST12140 engineered from strain ST11943 to additionally express a C-28 sterol methyltransferase under the control of a weak yeast promoter. Error bars represent ± 1 standard deviation Asterix (*) represents trace amount of β-sitosterol where value is below limit of detection for quantification;

Figure 18B shows sterol mixture production of a *Y. lipolytica* strain ST12178, engineered from strain ST12140 by additional introduction of expression of the delta-24(25) sterol reductase of *S. lycopersicum* from a codon -optimise coding sequence under the control of the PrGPAT promoter. Again error bars represent ± 1 standard deviation. Asterix (*) represents trace amount of β-sitosterol below the limit of detection for quantification.

Figure 19 shows time course of total sterol production, DCW and glycerol addition during fed-batch fermentation of strain ST11064 in a 250ml bioreactor.

Figure 20 shows the time course of sterol production, DCW, OD600, glucose concentration and glucose addition during fed-batch fermentation of ST4842 in a 5-L bioreactor. Error bars represent ± 1 standard deviation.

Figure 21: shows the time course of sterol production, DCW, OD600, glucose concentration and glucose addition during fed-batch fermentation of ST11005 in a 5-L bioreactor. Error bars represent ± 1 standard deviation.

Figure 22: shows the time course of sterol production, DCW, OD600, glucose concentration and glucose addition during fed-batch fermentation of ST121785 in a 5-L bioreactor. Error bars represent ± 1 standard deviation.

Figure 23 shows brood production over the course of three months in managed honeybee colonies provided with artificial diets supplemented with engineered yeast biomass in the form of a dried powder.

Figure 24 shows provision and consumption of artificial diets provided to the same honeybee colonies.

Detailed description

Yeast strains for use in providing yeast of the invention

"Oleaginous yeast" refers to yeast that can naturally accumulate more than 20% of their dry cell weight (DCW) as lipid and are of the *Dikarya* subkingdom of fungi. Oleaginous yeast includes organisms such as *Yarrowia lipolytica*, *Cryptococcus albidus*, *Lipomyces lipofera*, *Lipomyces starkeyi*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*,, *Trichosporon pullulan* and *Cutaneotrichosporon oleaginosus*.

Yeast engineered in accordance with the invention may be selected from any of the above-noted oleaginous yeast species. Preferably, the yeast cells are *Yarrowia lipolytica* or *Rhodotorula glutinis*. Most preferably the yeast is of the *Yarrowia lipolytica species*.

Yarrowia lipolytica is dimorphic yeast and belongs to the *Hemiascomycetes*. The entire genome of *Yarrowia lipolytica* is known. *Yarrowia* species is aerobic and considered to be non-pathogenic. *Yarrowia* is efficient in using hydrophobic substrates (e.g. alkanes, fatty acids, oils) and can grow on sugars. It has a high potential for industrial applications. *Yarrowia lipolytica* can accumulate lipid content to approximately 40% of its dry cell weight and is a model organism for lipid accumulation and remobilization.

For engineering of an oleaginous yeast strain of the invention where provision of a sterol surrogate is employed to ease required gene knock out in the ergosterol pathway, as indicated above, the oleaginous yeast ahead of engineering to modify sterol production (sometimes referred to as the platform strain) may be any oleaginous yeast where such knock out results in undesirable growth deficiency or no growth on a standard growth medium as defined above. As also noted above, such a *Y. lipolytica* yeast as preferred may be derived, for example, from the *Y. lipolytica* W29 strain Y-63746 (designated in Table 5 as ST4842 and available from the ARS culture collection) and is exemplified herein by the strain ST9100. ST9100 has a genotype:

MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt- >ERG12 IntC_3-SeACS<-PrGPDPrTefInt->YlACL1 IntD_1-IDI1<- PrGPD-PrTefInt->ERG20 (Arnesen et al (2020) *ibid)*.

It exhibits increased squalene biosynthesis and has other genotype characteristics which rendered it of particular interest for engineering for non-native sterol production.

Ku70p is implicated in DNA double-stranded break repair by non-homologous end-joining in Y. lipolytica. Thus deletion of the *ku70* gene in ST9100 promotes DNA double-stranded break repair by homologous recombination, allowing for easier genomic manipulation. A *Y. lipolytica* codon-optimized Cas9 gene from *Streptococcus pyogenes* is integrated into the *ku70* locus under control of the Tef promoter and terminator, using a dsdA marker cassette which allows growth on D-serine and can be used for selection. The integrated Cas9 gene enables efficient CRISPR/Cas9-mediated genome editing. Upregulation of a number of enzymes has been shown to increase terpenoid production in *Y. lipolytica*. The primary precursor of sterols is acetyl-CoA. The acetyl CoA pool can be increased by overexpression of the native ATP citrate lyase 1 (*ACL*) and the *Salmonella enterica* acetyl-CoA synthetase (*SeACS*). The enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG*) catalyses a key rate-limiting step of the melavonate pathway and HMG overexpression upregulates this pathway. The melavonate pathway feeds into the farnesyl pyrophosphate biosynthesis pathway. Overexpression of mevalonate kinase (*ERG12*), isopentyl diphosphate isomerase (*IDI*) and farnesyl diphosphate synthase (*ERG20*) increase flux toward farnesyl pyrophosphate (FPP). FPP is converted to squalene, which is the first intermediate of the sterol biosynthesis pathway.

It will be appreciated however that other oleaginous yeast may be alternatively similarly desirably engineered to provide yeast according to the invention expressing a sterol surrogate, e.g. other oleaginous yeast which exhibit increased squalene production above wild-type, especially other such *Y. lipolytica* which may be engineered also from *Y. lipolytica* W29 strain Y-63746 (ST4842) or any of ST6512, ST8980, ST9027 and ST9100. The starting platform yeast for introduction of the required heterologous genes(s) may desirably have all the modified genotype features of ST9100 compared with *Y. lipolytica* W29 strain Y-63746 as set out above. Some or all of these may desirably be retained in the final engineered yeast strain consistent with maintaining the desired non-native sterol production as further discussed below.

Gene deletion

Yeast of the invention may have a reduced ability to produce ergosterol or are incapable of producing ergosterol. Ergosterol, a 5,7,22-triene sterol, is the most abundant sterol in fungal cell membranes, where it regulates permeability and fluidity. Yeast ergosterol is synthesized through a highly conserved and complex pathway that can be divided into three modules. The first module is conserved across all eukaryotes and results in the formation of mevalonate from acetyl-coenzyme A (acetyl-CoA) by acetyl-CoA C-acetyltransferase (ERG10), formation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) by HMG-CoA synthase (ERG13) and reduction of HMG-CoA by HMG-CoA reductases (HMGR) Hmg1 and Hmg2 (Hmg1/2). The second module is carried out in the vacuole and involves the formation of farnesyl pyrophosphate (farnesyl-PP) by mevalonate kinase (ERG12), phosphomevalonate kinase (ERG8), mevalonate pyrophosphate decarboxylase (Mvd1/ERG19), isopentenyl diphosphate isomerase (Idi1) and farnesyl pyrophosphate synthetase (ERG20).

The third module or late pathway involves ergosterol synthesis itself through consecutive reactions that mainly occur in the endoplasmic reticulum (ER) membrane. Firstly, two molecules of farnesyl-PP are used by squalene synthase (ERG9) to form squalene, which is the precursor of all steroids.

Secondly, squalene is converted into lanosterol by the consecutive action of the squalene epoxidase (ERG1) and lanosterol synthase (ERG7). Lanosterol is transformed to zymosterol through a complex process involving various demethylation, reduction and desaturation reactions catalyzed by lanosterol 14-α-demethylase (ERG11, also known as Cyp51), C-14 reductase (ERG24) and C-4 demethylation complex which includes sterol C-4 methyloxidase (ERG25), sterol C-3 dehydrogenase (ERG26) and sterol C-3 ketoreductase (ERG27). ERG28 and ERG29 are likely to function in the C-4 demethylation complex reaction.

As seen in Figure 2, sterol C-24 methyltransferase (ERG6) converts zymosterol into fecosterol, followed by the formation of episterol by sterol C-8 isomerase (ERG2), which is desaturated and reduced by sterol C-5 desaturase (ERG3) to form 5, 7, 24(28)-ergostatrienol. This is then desaturated by sterol C-22 desaturase (ERG5) to 5, 7, 22, 24(28)-ergostatetraenol which is finally reduced by sterol C-24 reductase (ERG4) to ergosterol.

A yeast of the invention may be made incapable of producing ergosterol by attenuating activity of, or deleting, any one of the enzymes utilised in the third module or late pathway of ergosterol production. For example by attenuating activity of, or deleting, any one or more of sterol C-24 methyltransferase (ERG6), sterol C-8 isomerase (ERG2), sterol C-5 desaturase (ERG3), sterol C-22 desaturase (ERG5), and/or sterol C-24 reductase (ERG4). Commonly however, disruption of ergosterol production will be by attenuating activity of, or deleting, endogenous sterol C-22 desaturase enzyme (ERG5), delta-24 sterol reductase enzyme (ERG4) and/or sterol C-24 methyltransferase (ERG6). One or more of ERG4, ERG5 and ERG6 expression may be attenuated or deleted depending on the desired non-native sterol production desired as exemplified herein and illustrated by Figures 4 to 9.

As indicated above, a yeast of the invention may include replacement of ERG4 with a plant delta-24 sterol reductase (i.e. DWF1). That is to say that the yeast provided herein may include an attenuated or deleted ERG4 and include a heterologous nucleic acid encoding a plant delta-24(28) sterol

reductase (DWF1). The use of a plant delta-24 sterol reductase may be chosen to enable production of plant epimers of non-native sterols which may be beneficial for certain uses and applications such as insect (e.g. bee) foods.

The term attenuating or reducing activity are used to refer to a change in activity of an enzyme that leads to a level of the enzyme product below detectable levels or to levels lower than that seen in a reference or corresponding wild type strain. Attenuation or reduction of activity of an enzyme may be achieved by the use of genetic modification in order to inactivate or delete a gene or part of a gene encoding the protein. Suitable methods for reducing or preventing activity of a protein are well-known.

The terms “deletion,” “deleted,” “knockout,” and “knocked out” can be used interchangeably to refer to an endogenous gene that has been manipulated to no longer be expressed in an yeast of the invention.

A deletion can mean that at least part of the subject nucleic acid sequence is lost, but a deletion can also be accomplished by disrupting a gene through, for example, the insertion of another sequence (e.g. a selection marker), or a combination of deletion and insertion, but a deletion can also be performed by other genetic modifications. A deletion can mean that the gene no longer produces its functional gene product or, in various embodiments, that the gene produces less than 20% or less than 10% or less than 5% or less than 1% of its functional gene product versus production without the deletion under standard culturing conditions. The terms deletion cassette or vector and disruption cassette or vector are used interchangeably and refer to nucleic acid constructs that are inserted into a yeast of the invention to delete to attenuate a gene and therefore the gene product thereof such as the encoded enzyme

Required gene deletion or attenuation may be achieved using CRISPR-Cas/ gRNA based technology. Such technology may be used to replace the target gene with a selectable marker such as an antibiotic resistance gene. Deletion of the target gene then can be confirmed by exposing the modified yeast to the corresponding antibiotic.

After confirmation that the target gene has been deleted the selectable marker can be subsequently removed for example by use of a Cre-recombinase episomal system.

Other suitable methods include site directed mutagenesis, site specific nuclease based methods and/or homologous recombination.

Yeast of the invention may at least exhibit attenuated activity or deletion of an endogenous sterol C-22 desaturase enzyme (ERG5). Yeast of the invention may exhibit attenuated activity or deletion of endogenous delta-24 sterol reductase enzyme (ERG4). Yeast of the invention may exhibit attenuated activity or deletion of endogenous sterol C-24 methyl-transferase (ERG6). That is to say that yeast of the invention may include attenuated activity or deletion of endogenous sterol C-22 desaturase enzyme (ERG5), attenuated or deleted endogenous delta-24 sterol reductase enzyme (ERG4), and/or attenuated or deleted endogenous sterol C-24 methyltransferase enzyme (ERG6). A yeast of the invention as illustrated by embodiments exemplified herein may have deletion or attenuation of ERG5 and deletion or attenuation of one of ERG4 and ERG6.

The detrimental effects of attenuation or deletion of one or more of the above mentioned enzymes or genes may be increased in oleaginous yeast due to aerobic yeasts lacking sterol transporters that anaerobic yeast such as *Saccharomyces* and other yeasts possess which enable them to acquire sterols from their culture media.

Provision of a sterol Surrogate

As indicated above, it has been found by the inventors that to enable appropriate diversion of sterol biosynthetic flux from normal ergosterol synthesis in an oleaginous yeast to enable desired non-native sterol production, it may be necessary to provide intracellularly a sterol surrogate. Such provision of a sterol surrogate is called for whenever a parent strain for desired gene knock out in the ergosterol pathway is incompatible with adequate cell growth for useful non-native sterol production. This has first been observed with the known *Y. lipolytica* strain ST9100 which has a genotype that might otherwise be considered well-designed for non-native sterol production but as noted above can be expected to be a problem with other engineered oleaginous yeast strains.

As used herein, the term "sterol surrogate" refers to a heterologous compound that is utilised by a yeast to compensate for detrimental growth effect of disruption of the normal sterol biosynthesis pathway to ergosterol. It should enable deletion of any of the ERG4 gene, ERG5 gene and ERG6 genes which otherwise precludes adequate cell growth. It will be provided intracellularly, possibly at low level, for its required compensatory purpose. The inclusion of a sterol surrogate will help improve growth of a yeast that has reduced production of ergosterol or is incapable of producing ergosterol. In addition, the sterol surrogate may increase the production of exogenous (non-native) desired sterols.

A sterol surrogate may preferably be provided by expression of a heterologous nucleic acid encoding an enzyme for its direct production and as noted above will be controlled so that production of the desired non-native sterols can be achieved. For this reason, expression of the sterol surrogate may be under the control of a weak promoter in the chosen yeast cells such as the PrGPAT promoter or a functionally equivalent weak yeast promoter.

The sterol surrogate may preferably be tetrahymanol. Tetrahymanol is a pentacyclic triterpenoid having a 3beta- (21alpha-) hydroxy-substituted gammacerane structure. Other suitable compounds having similar structure and properties may also be used.

Without being bound by theory, tetrahymanol has the advantage of being better tolerated in the yeast membrane than other sterol surrogates and therefore may reduce toxicity of heterologous sterol overproduction or toxic intermediate production.

Tetrahymanol can be produced from squalene in a single, oxygen-independent cyclization reaction catalysed by a tetrahymanol synthase referred to by the Enzyme Commission (EC) number 4.2.1.123. For example, the tetrahymanol synthase may be a squalene-tetrahymanol cyclase.

Thus, a yeast of the invention may be provided with a heterologous nucleic acid sequence encoding a tetrahymanol synthase such as a squalene-tetrahymanol cyclase. The squalene-tetrahymanol cyclase may be the *Tetrahymena thermophilia* squalene-tetrahymanol cyclase (GenBank ascension number XP_001026696.2) or a functional variant thereof, preferably encoded by a codon-optimised sequence

and preferably under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter.

Alternatively, the sterol surrogate may be a hopanoid. Hopanoids are a diverse group of pentacyclic triterpenoid lipids mainly produced by bacteria. For example, the sterol surrogate may be hopene. Hopene can be produced from squalene by a squalene-hopene cyclase..

Thus, a yeast of the invention may be provided with a heterologous nucleic acid sequence encoding a squalene-hopene cyclase. For example the squalene-hopene cyclase may be the *Schizosaccharomyces japonicus* squalene-hopene cyclase or a functional variant thereof

Thus, by way of example, there is provided an oleaginous yeast comprising an attenuated or deleted endogenous sterol C-22 desaturase (ERG5) and at least one heterologous nucleic acid sequence encoding a squalene-tetrahymanol cyclase or a squalene-hopene cyclase.

Also provided is an oleaginous yeast comprising an attenuated or deleted endogenous sterol C-22 desaturase (ERG5) and an attenuated or deleted endogenous delta-24 sterol reductase (ERG4) and at least one heterologous nucleic acid sequence encoding a squalene-tetrahymanol cyclase or a squalene-hopene cyclase.

Also provided is an oleaginous yeast comprising an attenuated or deleted endogenous sterol C-22 desaturase (ERG5), preferably a deleted ERG5, an attenuated endogenous delta-24 sterol reductase which is either an attenuated ERG4 or an attenuated plant DWF1 provided by expression of a coding sequence, and at least one heterologous nucleic acid encoding a squalene-tetrahymanol cyclase or a squalene-hopene cyclase.

Also provided is an oleaginous yeast comprising an inactivated or deleted endogenous sterol C-22 desaturase (ERG5), an attenuated or deleted endogenous sterol C-24 methyl-transferase (ERG6) and at least one heterologous nucleic acid encoding a squalene-tetrahymanol cyclase or a squalene-hopene cyclase.

Also provided is an oleaginous yeast comprising an attenuated or deleted endogenous sterol C-24 methyl-transferase (ERG6) and at least one heterologous nucleic acid encoding a squalene-tetrahymanol cyclase or a squalene-hopene cyclase.

Such use of a sterol surrogate may allow for the yeast to maintain a growth rate similar to that of a wild type or reference yeast ahead of gene deletion.

As now further discussed below a yeast as above which (a) has reduced production of ergosterol production compared with a wild-type oleaginous yeast or is incapable of producing ergosterol and (b) is capable of expressing a sterol surrogate may additionally have incorporated one or more expressible heterogeneous genes whereby it is capable of producing one or more desired non-native sterols selected from one or more of campesterol, 24-methylenecholesterol, cholesterol, desmosterol, β-sitosterol, isofucosterol and stigmasterol. As indicated above, of especial interest, for example, is production of 24-methylenecholesterol either alone or in combination with one or more other sterols, e.g. campesterol and cholesterol.

Heterologous sterol encoding nucleic acid sequences

Yeast of the invention may include one or more heterologous nucleic acids encoding one or more of a delta-7 sterol reductase enzyme, a delta-24 sterol reductase enzyme, sterol C-28 methyltransferase enzyme and a sterol C-22 desaturase enzyme (see Table 1).

Heterologous enzymes which may be introduced into an oleaginous yeast, e.g. a *Y. lipolytica* yeast, to provide an engineered yeast according to the invention may include, for example one or more of the following:

a. a delta-7 sterol reductase enzyme selected from the group consisting of:
   Legionella drancourtii delta-7 sterol reductase;
   Ectocarpus siliculosus delta-7 sterol reductase;
   Candidatus Protochlamydia amoebophila delta-7 sterol reductase;
   Coccomyxa subellipsoidea delta-7 sterol reductase;
   Glycine soja delta-7 sterol reductase;
   Tetraselmis sp GSI018 delta-7 sterol reductase
   Solanum tuberosum delta-7 sterol reductase;
   Danio rerio delta-7 sterol reductase
   Mortierella verticillata delta-7 sterol reductase
   Waddlia chondrophila delta-7 sterol reductase and functional variants thereof; and/or
b. a delta-24 sterol reductase selected from the group consisting of:
   Danio rerio delta-24 sterol reductase;
   Bombyx mori delta-24 sterol reductase;
   Penaeus vannamei delta-24 sterol reductase;
   Aedes aegypti delta-24 sterol reductase;
   Gallus gallus delta-24 sterol reductase;
   Mus musculus delta-24 sterol reductase;
   Xenopus tropicalis delta-24 sterol reductase
   Solanum lycopersicum delta-24 sterol reductase;
   Notechis scutatus delta-24 sterol reductase; and/or
   Amblyraja radiata delta-24 sterol reductase;
   Arabidopsis thaliana delta-24 sterol reductase;
   Solanum tuberosum delta-24 sterol reductase;
   Arachis duranensis delta-24 sterol reductase;
   Selaginella moellendorffii delta-24 sterol reductase;
   Capsicum chinense delta-24 sterol reductase;
   Artemisia annua delta-24 sterol reductase;
   Helianthus annuus delta-24 sterol reductase;
   Cocos nucifera delta-24 sterol reductase;
   Triticum urartu delta-24 sterol reductase;
   Gracilariopsis chorda delta-24 sterol reductase;
   Capsella rubella delta-24 sterol reductase
   Ajuga reptans delta-24 sterol reductase and functional variants thereof; and/or
c. a sterol C-28 methyltransferase enzyme selected from the group consisting thereof:
   Cucurbita pepo sterol C-28 methyltransferase;
   Eutrema salsugineum sterol C-28methyltransferase;
   Arabadopsis thaliana sterol C-28 methyltransferase;
   Morus notabilis sterol C-28 methyltransferase;
   Amborella trichopoda sterol C-28 methyltransferase;
   Creolimax fragrantissima sterol C-28 methyltransferase;
   Ulva mutabilis sterol C-28 methyltransferase;

Rhodamnia argentea sterol C-28 methyltransferase;
Chenopodium quinoa sterol C-28 methyltransferase;
Glycine soja sterol C-28 methyl-transferase and functional variants thereof; and/or

d. a sterol C-22 desaturase enzyme which is:
Cytochrome P450 710A1 or a functional variant thereof.

As noted above, both *Solanum tuberosum* and *Solanum lycopersicum* possess two delta-24 sterol reductase variants, one catalyses delta-24(25) reduction and the other catalyses delta-24(28) reduction (see Table 1). One or more heterologous coding sequences for a delta- 24(28) sterol reductase and/or one or more heterologous coding sequences for a delta-24(25) sterol reductase may be expressed in a yeast of the invention.

As indicated above, where it is desired to maintain the same stereochemistry at a C-24 position of a sterol as observed in plants, e.g. in production of campesterol for some applications, then the yeast ERG4 gene encoding the yeast delta-24 sterol reductase will be substituted by a plant delta-24(28) sterol reductase, i.e. a DWF1 enzyme such as the *Arabidopsis thaliana* delta-24(28) sterol reductase (see Table 1). For production of the plant epimer of campesterol together with 24-methylenecholeserol in a *Y. lipolytica* of the invention lacking ERG5 and ERG4, expression of the *Solanum tuberosum* delta- 24(28) sterol reductase may, for example be preferred as illustrated herein by engineered *Y. lipolytica* strain ST11064 and strains derived therefrom.

Desirably, each heterologous enzyme incorporated in a yeast of the invention will be encoded by a codon-optimised sequence for expression in yeast, more desirably for use in the selected host species, e.g. *Y. lipolytica.* Appropriate codon-optimised sequences for all the above heterologous gene categories for use in engineering a *Y. lipolytica* strain are provided in Table 4 below.

For strong expression, the known PrTEFintron promoter may for example preferably be selected or a functionally equivalent promoter; i.e. a promoter that provides at least substantially the same expression or higher. However, as previously noted above, in some instances heterologous enzyme expression will be required or desirable under the control of a weaker promoter, e.g. the PrDGA1 or PrGPAT promoter as disclosed in Holkenbrink et al (2018) *ibid* or a functionally equivalent weak yeast promoter.

Delta-7 sterol reductase enzymes (EC 1.3.1.21) are involved in the production of cholesterol by reduction of the C7-C8 double bond of 7-dehydrocholesterol (7-DHC) and are capable of converting:

7-dehydrodesmosterol to desmosterol;
5-dehydroepisterol to 24-methylenecholesterol; and
5-dehydroavenasterol to isofucosterol.

The delta-7 sterol reductase may be an animal delta-7 sterol reductase such as DHCR7 or a plant delta-7 sterol reductase such as DWF5.

As noted above, the delta-7 sterol reductase enzyme may be selected from: *Legionella drancourtii* delta-7 sterol reductase; *Ectocarpus siliculosus* delta-7 sterol reductase; *Candidatus Protochlamydia amoebophila* delta-7 sterol reductase; *Coccomyxa subellipsoidea* delta-7 sterol reductase; *Glycine*

soja delta-7 sterol reductase; *Tetraselmis sp GSl018* delta-7 sterol reductase; *Solanum tuberosum* delta-7 sterol reductase, *Danio rerio* delta-7 sterol reductase, *Mortierella verticilliata* delta-7 sterol reductase, *Waddlia chondrophila* delta-7 sterol reductase and functional variants thereof. For expression in an engineered *Y. lipolytica* of the invention, expression of the delta-7 reductase of *Tetraselmis* sp. GS1080 or *Legionella drancourtii* may for example be favoured. However, it will be recognised that use of other delta-7 sterol reductases is not excluded. Expression in an engineered *Y. lipolytica* of the invention may desirably be under the control of the PrTEFintron promoter or a functionally equivalent strong promoter.

Delta-24 sterol reductase enzymes act on a range of steroids with a 24(25)-double bond or 24(28)-double bond and are capable of converting:

desmosterol to cholesterol;
24-methylenecholsterol to campesterol; and
isofucosterol to sitosterol.

Where expression of a delta-24(25) sterol reductase is required this may be an animal delta-24(25) sterol reductase such as DHCR24 or a plant delta-24(25) reductase such as SSR2. As previously discussed, a plant delta-24(28) sterol reductase (DWF1) will be utilised to substitute for yeast ERG4.

As noted above, a delta-24 sterol reductase for expression in a yeast of the invention may be selected from or be a functional variant of: *Danio rerio* delta-24 sterol reductase; *Bombyx mori* delta-24 sterol reductase; *Penaeus vannamei* delta-24 sterol reductase*; Aedes aegypti* delta-24 sterol reductase; *Gallus gallus* delta-24 sterol reductase *Mus musculus* delta-24 sterol reductase; *Xenopus tropicalis* delta-24 sterol reductase; a *Solanum lycopersicum* delta-24 sterol reductase; *Notechis scutatus* delta-24 sterol reductase; *Amblyraja radiata* delta-24 sterol reductase; *Arabidopsis thaliana* delta-24 sterol reductase; a *Solanum tuberosum* delta-24 sterol reductase; Arachis duranensis delta-24 sterol reductase; *Selaginella moellendorffii* delta-24 sterol reductase; *Capsicum chinense* delta-24 sterol reductase; *Artemisia annua* delta-24 sterol reductase; *Helianthus annuus* delta-24 sterol reductase; *Cocos nucifera* delta-24 sterol reductase; *Triticum urartu* delta-24 sterol reductase; *Gracilariopsis chorda* delta-24 sterol reductase; *Capsella rubella* delta-24 sterol reductase; or *Ajuga reptans* delta-24 sterol reductase.

However, it is further emphasised that for production of plant sterols, such as campesterol, beta-sitosterol and stigmasterol, a plant delta-24(28) sterol reductase is required to replace yeast ERG4 and thereby preferably achieve the correct stereochemistry. Stereochemistry around C-24 in ergosterol is different to that of plant sterols, due to differences in the respective delta-24 sterol reductase enzymes. As such, sterols such as campesterol produced in previous work using animal or yeast delta-24 sterol reductases are the C-24 β epimer forms of the sterols. For example, ERG4 produces campesterol in the C-24β configuration (S). The epimers may be indistinguishable by gas chromatography-mass spectroscopy but the differences may not be trivial. For example, as previously noted, the moulting hormone Makisterone A is produced from campesterol in honeybees. Provision of the 24 β epimer of campesterol would lead to the production of epi-Makisterone A, which may not function in the same way (e.g. some insects use only the plant epimer form). By deleting the native ERG4 and expressing a heterologous plant delta-24(28) sterol reductase, the correct plant

stereochemistry at C-24 (24α configuration (R)) of for example campesterol can be achieved (see Figure 16). As previously noted, by way of example of a preferred enzyme for this purpose in an engineered *Y. lipolytica* of the invention is the delta-24(28) sterol reductase of *Solanum tuberosum.* Expression will generally be under the control of a weak yeast promoter such as the PrDGA1 promoter or a functionally equivalent weak yeast promoter.

Sterol C-28 methyltransferase enzymes (EC 2.1.1.143) also known as 24-methylenesterol C-methyltransferase acts in the second methylation step of plant sterol biosynthesis and is capable of converting:

episterol to delta 7-avenasterol; and

5-dehydroepisterol to 5-dehydroavenasterol.

The sterol C-28 methyltransferase enzyme employed in yeast of the invention may be a plant sterol C-28 methyltransferase enzyme such as SMT2.

As noted above, the sterol C-28 methyltransferase enzyme may be selected from or a functional variant of: *Cucurbita pepo* C-28 methyltransferase; *Eutrema salsugineum* C-28 methyltransferase; *Arabadopsis thaliana* C-28 methyltransferase; *Morus notabilis* C-28 methyltransferase; *Amborella trichopoda* C-28 methyltransferase; *Creolimax fragrantissima* C-28 methyltransferase; *Ulva mutabilis* C-28 methyl-transferase; *Rhodamnia argentea* C-28 methyl-transferase; *Chenopodium quinoa* C-28 methyl-transferase or *Glycine soja* C-28 methyltransferase. Use of more than one such enzyme may be favoured, e.g. one, two or three selected from the C-28 sterol methyltransferases of *Chenopodium quinoa, Arabidopsis thaliana* and *Amborella trichopoda.*

Sterol C-22 desaturase enzymes (EC1.14.19.41) act on sitosterol and 24-epi-campesterol, producing stigmasterol and brassicasterol respectively. The sterol C-22 desaturase enzyme may be Cytochrome P450 710A1.

Table 1 provides details of the enzymes mentioned above as well as the names of the enzymes in yeast, plants and animals.

Optional additional genotype features

Optionally, an oleaginous yeast of the invention may exhibit reduced activity of at least one or more of endogenous:

PAH1 (Mg2+-dependent phosphatidate phosphatase), Bts1 (geranylgeranyl pyrophosphate synthase), GDH1 (NADP-specific glutamate dehydrogenase 1), Are1 (sterol O-acyltransferase), Say1 (steryl acetyl hydrolase), Sds23, and/or Ins1. For example, PAH1 gene knock-out may be deemed desirable to increase the amount of membranes and capacity for sterol accumulation as illustrated by *Y. lipolytica* strain ST11197 referred to herein (an ERG5/ PAH1 knock out derived from ST9100 and expressing a squalene-tetrahymanol cyclase gene and *Ectocarpus siliculosis* delta-7 sterol reductase). This strain enabled production of campesterol at as high as 41.5 mg/g DCW with 72hrs culture with glucose as the sole carbon source.

Optionally, an oleaginous yeast of the invention may exhibit increased activity of at least one or more of:

HMG1 (3-hydroxy-3-methylglutaryl-coenzyme A reductase 1), truncated HMG1, UPC2 (sterol uptake control protein 2), Ecm22 (sterol regulatory element binding protein), Erg1 (squalene epoxidase), Erg3 (sterol C-5 desaturase), Erg7 (lanosterol synthase), Erg8 (phosphomevalonate kinase), Erg9 (squalene synthase), Erg10 (acetyl-CoA C-acetyltransferase), Erg11 (lanosterol 14-α-demethylase), Erg12 (mevalonate kinase), Erg13 (HMG-CoA synthase), Erg19 (mevalonate pyrophosphate decarboxylase), Erg20 (farnesyl pyrophosphate synthetase), Erg25 (sterol C-4 methyloxydase), Erg26 (sterol C-3 dehydrogenase), Erg27 (sterol C-3 ketoreductase), IDI (isopentenyl diphosphate isomerase), Acl (ATP-citrate lyase), Pot1 (3-ketoacyl-CoA thiolase), Pat1 (peroxisomal acetoacetyl-CoA thiolase), Pex10 (peroxisomal membrane E3 ubiquitin ligase), GDH2 (NAD(+)-dependent glutamate dehydrogenase), G6PD1 (Glucose-6-phosphate 1-dehydrogenase 1), Are1 (sterol O-acyltransferase), Are2 (acyl-CoA:sterol acyltransferase), Atf2 (alcohol acetyltransferase), Acs1 (acetyl-coA synthetase), SSD1 (protein SSD1), YBP1 (YAP1-binding protein), Sre1 (sterol regulatory element-binding protein 1) and/or Css1 (secreted protein CSS1) or variants thereof.

As noted above, stereochemistry around C-24 in ergosterol is different to that in plant sterols. Thus, where it is desired to obtain a non-native sterol with plant stereochemistry at C-24, e. g. the 24α epimer of campesterol, deletion of the endogenous delta-24 sterol reductase enzyme (ERG4) will be accompanied by expression of a heterologous nucleic acid encoding a plant delta-24 sterol reductase enzyme (DWF1). Thus whilst production of campesterol may be achieved by just deletion or attenuation of the ERG5 gene and expression of a heterologous gene for a delta-7 sterol reductase, e.g. a plant DWF5, preferably in some instances, the yeast ERG4 gene may additionally be knocked out and a DWF1 expressed whereby campesterol production is maintained but as the plant epimer.

Culturing for non-native sterol production

For production of the one or more desired sterols by a yeast of the invention, culturing may be carried out in any culture medium under conditions suitable for expressing the one or more of the heterologous nucleic acids as described herein. However, preferably a single simple carbon feed source will be provided, e.g. glucose. Thus for example culturing may be in conventional yeast extract peptone dextrose (YPD) medium supplemented with glucose at 30°C. By such culturing of a yeast of the invention, as exemplified herein, a desired sterol or mixture of sterols may be attained at a production level of at least 9-10 mg/g of dry cell weight (DCW), for example at least 25 mg/g DCW, possibly at least 30 or 40mg/ g DCW or even higher, e.g. at least 45-50 mg/g.

By way of example, the invention provides an oleaginous yeast that is capable of producing commercially viable levels of 24-methylenecholesterol having:

an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and delta-24 sterol reductase enzyme (ERG4);

a heterologous nucleic acid for provision intracellularly of a sterol surrogate, e.g. encoding a squalene-tetrahymanol cyclase and

a heterologous nucleic acid encoding a delta-7 sterol reductase enzyme as described herein;

wherein the yeast is capable of producing 24-methylenecholesterol at a concentration of at least 9-10 mg/g DCW, possibly together with one or more additional non-native sterols, e.g. campesterol and cholesterol at measurable amounts, or greater than 25mg/g DCW. By means of such a yeast 24-methylenecholesterol concentrations of at least 30mg/g, 35mg/g, 40mg/g or 45- 50 mg/g may for example be achieved.

Preferred embodiments of engineered yeast of the invention are further discussed below with reference to Table 2 and the figures.

Preferred embodiments of yeast of the invention expressing a sterol surrogate

Table 2 provides a summary of different genetic modifications for producing specific sterols.

Now described are various preferred embodiments of yeast of the invention as discussed above where the yeast has reduced production of ergosterol compared with a wild-type oleaginous yeast or is incapable of producing ergosterol and this is coupled with intracellular provision of a sterol surrogate whereby the disruption of ergosterol biosynthesis, e.g. by gene deletion, is compensated for to aid growth. By way of example, for such preferred embodiments, the platform strain for such engineering, as illustrated by the exemplification, may be *Y. lipolytica* ST9100. However, as indicated above, it is envisaged that similar non-native sterol production may be achieved by the same strategy with other oleaginous yeast, e.g. other *Y. lipolytica* strains, which share with ST9100 knock out of any of the ERG4, ERG5 and ERG6 genes being incompatible with adequate cell growth, e.g. other such engineered yeast which have been modified to increase squalene synthesis compared to wild-type. Without being bound by theory, it has been postulated that this may be related to harmful accumulation of sterol intermediates but equally increased squalene synthesis may contribute to good non-native sterol production in final engineered strains of the invention.

A. Thus, in one preferred embodiment a yeast as described above expressing a sterol surrogate and engineered for production of one more non-native sterols comprising campesterol has:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5); and

(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme as described herein.

Preferably, the sterol surrogate may be provided by expression of a squalene- tetrahymanol cyclase under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter.

The yeast may further include an attenuated or deleted endogenous delta-24 sterol reductase enzyme (ERG4) and a heterologous nucleic acid encoding a plant delta-24(28) sterol reductase enzyme (DWF1). That is to say the yeast may have endogenous delta-24 sterol reductase enzyme (ERG4) replaced by a heterologous nucleic acid encoding a plant delta-24(28) sterol reductase enzyme (DWF1).

As seen in Figure 4, the yeast converts squalene to 5-dehydroepisterol using endogenous sterol C-24 methyltransferase (ERG6), sterol C-8 isomerase (ERG2) and sterol C-5 desaturase (ERG3). As the yeast lacks endogenous sterol C-22 desaturase (ERG5), the 5-dehydroepisterol is converted by the heterologous delta-7 reductase (DWF5) to 24-methylenecholesterol. Endogenous sterol C-24

reductase (ERG4) converts the 24-methylenecholesterol to campesterol. Alternatively, the final step is performed by a plant DWF1 substituting for the endogenous ERG4 to attain the plant 24α epimer (24(R)- campesterol).

By incorporating into a platform strain as above, e.g. *Y. lipolytica ST9100*, (i) ERG5 gene deletion facilitated by expression of a codon-optimised coding sequence for squalene-tetrahymanol cyclase of Tetrahymena thermophilia (TtSTC) under the control of a weak yeast promoter, e,g. PrGPAT and (ii) a codon-optimised coding sequence for expression of a delta-7 sterol reductase, e.g. *Coccomyxa subellipsoidea* delta -7 sterol reductase, *Ectocarpus siliculosus* delta-7 sterol reductase or the delta-7 sterol reductase gene variant of Tetraselmis sp. GSL08 , under the control of the PrTEFintron promoter or a functionally equivalent yeast promoter, production of campesterol has been shown to be achievable at a level of at least 30mg/g DCW, e.g. about 40mg/g DCW (culturing at 30°C in conventional YPD medium with glucose). Such engineering may be combined with *PAH1* gene knock-out to further boost campesterol production. See Example 3 and Figures 12 and 13. As indicated above, it may also be combined with substitution of yeast ERG4 by a plant DWF1.

Campesterol is an important precursor for steroid drugs such as progesterone, pregnenolone, and hydrocortisone. Campesterol is also a major precursor for the production of brassinosteroids which may have antiviral, antifungal, antiproliferative, antibacterial, neuroprotective and immunomodulatory properties in animals. In addition, campesterol may be used as a dietary additive. Campesterol may also be used in artificial dietary compositions for example for insects such as bees. Particularly, it is again noted that campesterol produced by a plant delta-24(28) sterol reductase enzyme may be useful in an artificial dietary composition for bees as the campesterol produced will be the plant epimer.

B. In another preferred embodiment a yeast as described above expressing a sterol surrogate and engineered for production of one more non-native sterols comprising desmosterol has:

(i) an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6); and
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme as described herein.

The yeast may further include an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or delta-24 sterol reductase enzyme (ERG4).

Preferably, again the sterol surrogate may be provided by expression of a squalene-tetrahymanol cyclase under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter

Preferably, the delta-7 sterol reductase encoded by the heterologous nucleic acid is an animal delta-7 sterol reductase, for example DHCR7. The chosen delta-7 sterol reductase, e.g. the delta-7 sterol reductase of *Legionella drancourtii* or another delta-7 sterol reductase as listed above, will preferably be expressed by a codon-optimised coding sequence e.g. a codon-optimised sequence for *Y. lipolytica* again under the control of the PrTEFintron promoter or a functionally equivalent yeast promoter.

As seen in Figure 6, as the yeast lacks an endogenous C-24 methyltransferase (ERG6) it is incapable of converting zymosterol to fecosterol. Instead zymosterol is converted by endogenous

sterol C-8 isomerase (ERG2) to cholesta-7,24-dienol, which is then converted to 7-dehydrodesmosterol by endogenous sterol C-5 desaturase (ERG3). This is then converted to desmosterol by the heterologous delta-7 reductase (DHCR7). Attenuation or deletion of endogenous sterol C-22 desaturase (ERG5) and endogenous sterol C-24 reductase (ERG4) activity may help to prevent or reduce the production of by-products as the yeast is incapable of acting on the 7-dehydrodesmosterol or desmosterol as well as incapable of producing ergosterol leading to production of desmosterol.

Desmosterol, also known as cholesta-5,24-dien-3β-ol, is an immediate precursor of cholesterol in the Bloch pathway of sterol synthesis and an abundant membrane lipid in specific types of organisms.

C. In another preferred embodiment a yeast as described above expressing a sterol surrogate and engineered for production of one more non-native sterols comprising cholesterol has:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted sterol C-24 methyltransferase (ERG6);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme as described herein and
(iii) a heterologous nucleic acid sequence encoding a delta-24 sterol reductase enzyme as described herein.

The delta-7 sterol reductase encoded by a heterologous nucleic acid may be an animal delta-7 sterol reductase, for example DHCR7, but a wide variety of enzymes with delta-7 sterol reductase activity may alternatively be employed. Preferably, the delta-24 sterol reductase encoded by a heterologous nucleic acid is an animal delta-24 sterol reductase, for example DHCR24.

By way of example, expression of the delta-7 sterol reductase of *Legionella drancourtii* may be combined with expression of the delta-24 sterol reductase of *Danio rerio* for cholesterol production in an engineered *Y. lipolytica* of the invention as illustrated by strain ST11829 described further herein (see Example 4). For this purpose, the heterologous coding sequences for both the delta-7 sterol reductase and delta-24 sterol reductase will desirably be expressed by codon-optimised coding sequences for *Y. lipolytica* under the control of the PrTEFintron promoter or a functionally equivalent yeast promoter.

Again, preferably, the sterol surrogate may be provided by expression of a squalene- tetrahymanol cyclase under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter.

As seen in Figure 7, as the yeast lacks an endogenous C-24 methyltransferase (ERG6) it is incapable of converting zymosterol to fecosterol. Instead zymosterol is converted by endogenous sterol C-8 isomerase (ERG2) to cholesta-7,24-dienol, which is then converted to 7-dehydrodesmosterol by endogenous sterol C-5 desaturase (ERG3). This is then converted to desmosterol by a heterologous delta-7 reductase (DHCR7). Desmosterol is then converted to cholesterol by a heterologous delta-24 sterol reductase (DCHR24). As the yeast lacks endogenous sterol C-22 desaturase (ERG5) it is incapable of acting on the 7-dehydrodesmosterol or desmosterol as well as incapable of producing ergosterol leading to production of cholesterol.

Cholesterol is an essential structural component of animal cell membranes as well as being a precursor for the biosynthesis of steroid hormones, bile acid and vitamin D.

D. In a further preferred embodiment a yeast as described above expressing a sterol surrogate and engineered for production of one or more non-native sterols comprising isofucosterol (either the Z isomer or the E-isomer, more commonly distinguished by naming as fucosterol) has:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme as described herein; and
(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme as described herein.

Preferably, the sterol C-28 methyltransferase encoded by a heterologous nucleic acid is a plant sterol C-28 methyl-transferase, for example SMT2 which produces solely isofucosterol as the Z plant isomer. As indicated above, to boost isofucosterol production in some instances it may be found preferable to employ more than one coding sequence for a sterol C-28 methyltransferase, e.g. 1 to 3 such coding sequences which may be the same or different. For production of isofucosterol in an engineered *Y. lipolytica* of the invention, such coding sequences will desirably be codon optimised for *Y.lipolytica* and under the control of for example a strong yeast promoter such as the PrTEFintron *or* PrGPD promoter

Preferably, the delta-7 sterol reductase encoded by a heterologous nucleic acid is a plant delta-7 sterol reductase, for example DWF5. For production of isofucosterol in an engineered *Y. lipolytica* of the invention, the parent starting strain may for example be a ΔERG5 ΔERG4 *Y.lipolytica* strain expressing the *Tetraselmis* sp. GSL018 delta-7 sterol reductase, again from a codon optimised sequence and under control of a PrTEFintron promoter or functionally equivalent promoter as illustrated by strain ST11064 and its further conversion to strains ST11803 and ST12108. For further discussion of such strain development for isofucosterol production, see again Example 4.

Again, preferably, the sterol surrogate may be provided by expression of a squalene- tetrahymanol cyclase under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter

As shown in Figure 8, squalene is converted to episterol and 5-dehydroepisterol by endogenous sterol C-24 methyltransferase (ERG6), sterol C-8 isomerase (ERG2) and sterol C-5 desaturase (ERG3). Episterol and 5-dehydroepisterol are then converted to delta 7-avenasterol or 5-dehydroavenosterol respectively by the heterologous sterol C-28 methyltransferase. Delta 7-avenasterol is also converted to 5-dehydroavenosterol by endogenous sterol C-5 desaturase (ERG3). 5-dehydroavenosterol is then converted to isofucosterol by the heterologous delta-7 sterol reductase. As the yeast lacks endogenous sterol C-24 reductase (ERG4) activity it is incapable of converting isofucosterol to sitosterol leading to production of isofucosterol.

Isofucosterol exhibits various biological therapeutic properties, including anticancer, antidiabetic, antioxidant, hepatoprotective, antihyperlipidemic, antifungal, antihistaminic, anticholinergic, antiadipogenic, anti-photodamaging, anti-osteoporotic, blood cholesterol reducing, blood vessel

thrombosis preventive and butyrylcholinesterase inhibitory activities. Isofucosterol may also be used in artificial dietary compositions for example for insects such as bees.

E. In a further preferred embodiment a yeast as described above expressing a sterol surrogate and engineered for production of one or more non-native sterols comprising beta-sitosterol has:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme as described herein; and
(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme as described herein.

The yeast may further include an attenuated or deleted endogenous delta-24 sterol reductase enzyme (ERG4) and a heterologous nucleic acid encoding a plant delta-24 sterol reductase enzyme (DWF1). That is to say the yeast may have endogenous delta-24 sterol reductase enzyme (ERG4) replaced by a heterologous nucleic acid encoding a plant delta-24 sterol reductase enzyme (DWF1). This provides a yeast that produces beta-sitosterol in the same configuration (epimer) as found in plants.

Preferably, the sterol C-28 methyltransferase encoded by the heterologous nucleic acid is a plant sterol C-28 methyl-transferase, for example SMT2. Again, it may be found favourable to provide more than one sterol C-28 methyltransferase, preferably under the control of a strong yeast promoter, e.g. a PrTEFintron promoter or PrGPD promoter in the case of an engineered *Y.lipolytica* host.

Preferably, the delta-7 sterol reductase encoded by the heterologous nucleic acid is a plant delta-7 sterol reductase, for example DWF5, e.g. the delta-7 sterol reductase variant of *Tetraselmis* sp. GSL018. The parent strain may for example conveniently be a campesterol-producing *Y. lipolytica* ΔERG 5 strain in which the delta-7 sterol reductase variant of *Tetraselmis* sp.GSL018 is expressed from a codon-optimised sequence under the control of a PrTEFintron promoter or functionally equivalent promoter.

Again, preferably, the sterol surrogate may be provided by expression of a squalene- tetrahymanol cyclase under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter

As shown in Figure 9, squalene is converted to episterol and 5-dehydroepisterol by endogenous sterol C-24 methyltransferase (ERG6), sterol C-8 isomerase (ERG2) and sterol C-5 desaturase (ErRG3). Episterol and 5-dehydroepisterol are then converted to delta 7-avenasterol or 5-dehydroavenosterol respectively by the heterologous sterol C-28 methyl-transferase. Delta 7-avenasterol is also converted to 5-dehydroavenosterol by endogenous sterol C-5 desaturase (ERG3). 5-dehydroavenosterol is then converted to isofucosterol by heterologous delta-7 reductase. Endogenous delta-24 sterol reductase (ERG4) (or a substituted plant delta-24 sterol reductase) then converts isofucosterol to beta-sitosterol.

Beta-sitosterol is most commonly used for lowering cholesterol levels and improving symptoms of an enlarged prostate (benign prostatic hyperplasia or BPH) in human subjects. As such beta-sitosterol may be used in food compositions. In addition, beta-sitosterol may be used in artificial dietary

compositions for example for insects such as bees. Beta-sitosterol is also a precursor for the production of brassinosteroids which may have antiviral, antifungal, antiproliferative, antibacterial, neuroprotective and immunomodulatory properties in animals.

F. As indicated above, of especial importance, for the first time there is now provided engineered yeast for production of 24-methylenecholesterol, either as a single sterol or as a component of a sterol mixture at a measurable amount. Thus, in a preferred embodiment a yeast as described above expressing a sterol surrogate and engineered for production of one or more non-native sterols comprising 24-methylenecholesterol has:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4); and
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme as described herein.

Preferably, the delta-7 sterol reductase encoded by the heterologous nucleic acid is a plant delta-7 sterol reductase, for example DWF5.

Again, preferably, the sterol surrogate may be provided by expression of a squalene- tetrahymanol cyclase under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter

As seen in Figure 5, if both the ERG5 and ERG4 genes are deleted, the yeast converts squalene to 5-dehydroepisterol using endogenous sterol C-24 methyltransferase (ERG6), sterol C-8 isomerase (ERG2) and sterol C-5 desaturase (ERG3). As the yeast lacks endogenous sterol C-22 desaturase (ERG5), the 5-dehydroepisterol is converted by the heterologous delta-7 reductase (DWF5) to 24-methylenecholesterol. As the yeast lacks sterol C-24 reductase (ERG4) activity, it is incapable of acting on the 24-methylenecholesterol as well as incapable of producing ergosterol leading to production of 24-methylenecholesterol.

24-methylenecholesterol may be used as a precursor for compounds such as withanolides which may have antimicrobial, anti-viral, anti-tumour, anti-arthritic, anti-aging, anti-inflammatory and neuroprotective properties. 24-methylenecholesterol may also be used for artificial dietary compositions, for example for insects such as bees.

24-methylenecholesterol has previously been recognised as an intermediate but has not previously been produced by any engineered yeast in useful manner and quantity. In contrast now provided are engineered yeast strains as indicated above which are able to provide 24-methylenecholesterol at high quantity, either as a single sterol or as part of sterol mixture containing one or more further non-native sterols in measurable amount, e.g. campesterol or campesterol and cholesterol.

By way of example of an especially preferred embodiment, by incorporating into a platform strain as above, e.g. *Y. lipolytica ST9100*, (i) both ERG5 gene deletion and ERG 4 gene deletion facilitated by expression of a coding sequence for a sterol surrogate such as preferably a codon-optimised coding sequence for squalene-tetrahymanol cyclase of Tetrahymena thermophilia (TtSTC) under the control of a weak yeast promoter, e,g. PrGPAT and (ii) a codon-optimised coding sequence for expression of a delta-7 sterol reductase, e.g. *Tetraselmis sp.* GSL018 delta-7 sterol reductase, under the control of

the PrTEFintron promoter or a functionally equivalent yeast promoter, production of 24-methylenecholesterol has been achieved at levels of more than 25mg/g DCW, e.g. as high as 40-50mg/g DCW. Using the *Tetraselmis s*p. GSL018 delta- 7 sterol reductase in such an engineered strain derived from ST9100, production of 24-methylenecholesterol has been achieved at about 48 mg/g DCW. Such high 24-methylenecholesterol production was not predictable from any prior art studies with *S. cerevisiae* and has been achieved by culturing without feeding any sterol precursor but employing a conventional yeast culturing medium (YPD medium) containing glucose at 30°C. See in Example 3 re strain ST11064 and Figure 14.

G. It will be appreciated however from above that there is equally interest for some applications in obtaining 24-methylenecholesterol as a major component of a sterol mixture, e.g. for incorporation into an artificial dietary composition for bees. In this case a platform strain as above, e.g. *Y. lipolytica* ST9100, may have an attenuated or deleted ERG5 gene, preferably a deleted ERG5 gene, and activity of ERG4 attenuated. This may be achieved by provision of the same coding sequence under the control of a weak promoter selected from the PrDGA1 promoter and functionally equivalent weak yeast promoters. Alternatively, the ERG4 gene may be substituted by a plant DWF1 coding sequence providing attenuated delta-24(28) sterol reductase activity under the control of a weak promoter such as the PrDGA1 promoter or a functionally equivalent weak promoter. In the presence also of expression of a coding sequence (preferably codon-optimised) for a delta-7 sterol reductase, e.g. *Tetraselmis* sp. GSL018 delta-7 sterol reductase, under the control of a stronger promoter, e.g. the PrTEFintron promoter or a functionally equivalent promoter, a sterol mixture comprising both 24-methylenecholesterol and campesterol in measurable amount can be attained. See Example 4. Moreover, if the yeast ER4G gene is substituted by a DWF1 gene, the campesterol component may importantly be obtained as the plant epimer. By way of example in providing such an engineered *Y. lipolytica* strain, expression of a delta-7sterol reductase, such as the Tetraselmis sp. GSL018 delta- 7 reductase under the control of the PrTEFintron promoter or a functionally equivalent strong yeast promoter may be combined with expression of the delta-24(28) sterol reductase gene from *Solanum tuberosum* (StSSR1) under the control of the PrDGA1 promoter or an equivalent weak yeast promoter as illustrated by strain ST11943 as described herein. By arranging for expression of the heterologous coding sequence for the delta-7 sterol reductase to be controlled by a strong yeast promoter and delta-24(28) sterol reductase expression to be controlled by a weaker promoter, a sterol mixture can be produced in which 24-methylenecholesterol is the dominant sterol, e.g. at above 15 mg/g DCW, e.g. about 18 mg/g DCW, but accompanied by useful, quantifiable campesterol, preferably as the plant epimer

Further incorporating a delta-24(25) sterol reductase under the control of a weak promoter, e.g. the PrGPAT promoter or a functionally equivalent promoter, may be preferred, e.g. introducing a codon-optimised coding sequence for the *S. lycoperscium* delta-24(25) sterol reductase under the control of the PrGPAT promoter or a functionally equivalent promoter. It has been shown that this can enable some 24-methylenecholesterol to be converted to cholesterol but with maintenance of 24-methylenecholesterol as the primary component of the attained sterol mixture with both measurable cholesterol and campesterol. Indeed, by such engineering of ST9100 as the platform strain 24-

methylenecholesterol has still been attained at about 9-10 mg/g DCW in a mixture with both measurable cholesterol and campesterol. See Example 4.

As indicated above, further introduction of a C-28 sterol methyltransferase under the control of a weak promoter, e.g. the *C. quinoa* C-28 sterol methyltransferase encoded by a codon-optimised nucleic acid sequence under the control of the PrGPAT promoter or a functionally equivalent promoter, may be further considered to obtain a mixture of sterols. Such engineered Y. lipolytica strains are exemplified by strains ST11362 and strains ST11541 as further described in Example 4. As further discussed below, it has been found possible to further engineer these strains to omit expression of the sterol surrogate, thereby providing strains ST11441 and ST11542 (see strain construction tables 19 and 20 in the exemplification).

The genes for expression of a delta- 24(25) sterol reductase and C-28 sterol reductase may be introduced in either alternative order as further illustrated by the strain construction table 21 for *Y.lipolytica* strain ST12178.

Thus as a preferred embodiment of an oleaginous yeast of the invention supplied with a sterol surrogate, there is provided such a yeast capable of producing a mixture of desired sterols comprising 24-methylenecholesterol, campesterol and cholesterol, optionally together with one or more further non-native sterols in detectable amount, wherein the oleaginous yeast comprises:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5), preferably deleted ERG5;

(ii) an attenuated delta-24 sterol reductase enzyme (ERG4) or ERG4 substituted by a plant DWF1 enzyme providing attenuated delta-24(28) sterol reductase activity, e.g. where the ERG4 gene coding sequence or plant DWF1 enzyme coding sequence is under the control of the PrDGA1 promoter or a functionally equivalent weak promoter;

(iii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. the delta-7 sterol reductase variant of *Tetraselmis* sp. GSL018, preferably under the control of a stronger promoter than employed for (ii), e.g. the PrTEFintron promoter or a functionally equivalent promoter;

(iv) a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, e.g. the delta-24(25) sterol reductase of *S. lycopersicum*, preferably under the control of the PrGPAT promoter or a functionally equivalent weak promoter

and optionally

(v) a heterologous nucleic acid sequence encoding a C-28 sterol methyltransferase, e.g. the C-28 sterol methyltransferase of *C. quinoa,* preferably under the control the PrGPAT promoter or a functionally equivalent promoter,

whereby said mixture of non-native sterols can be produced.

Alternatively, the heterologous nucleic acid sequence encoding the C-28 sterol methyltransferase may be provided and the heterologous nucleic acid sequence encoding the delta- 24(25) sterol reductase omitted or introduced subsequently.

By way of a particularly favoured oleaginous yeast of the invention supplied with a sterol surrogate, preferably tetrahymanol from expression of a squalene-tetrahymanol cyclase coding sequence, there is provided such a strain, preferably an engineered *Y. lipolytica*, which is capable of producing a mixture of non-native sterols comprising 24-methylenecholesterol, campesterol as the 24R plant epimer, cholesterol, isofucosterol and desmosterol, wherein the oleaginous yeast comprises:

(i) a deleted endogenous sterol C-22 desaturase enzyme (ERG5),

(ii) ERG4 substituted by a DWF1 enzyme providing attenuated delta-24(28) sterol reductase activity, e.g. the delta-24(28) sterol reductase (DWF1) of *Solanum tuberosum*, where said DWF1 enzyme is under the control of the PrDGA1 promoter or a functionally equivalent weak promoter;

(iii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. the delta-7 sterol reductase of *Tetraselmis* sp. GSL018, where said delta-7 sterol reductase is under the control of a stronger promoter than employed for (ii), e.g. the PrTEFintron promoter or a functionally equivalent promoter;

(iv) a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, e.g. the delta-24(25) sterol reductase of *S. lycopersicum*, preferably under the control of the PrGPAT promoter or a functionally equivalent weak promoter and

(v) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase, e.g. the sterol C-28 methyltransferase of *C. quinoa,* preferably under the control of the PrGPAT promoter or a functionally equivalent promoter,

whereby said mixture of non-native sterols can be produced.

Such an engineered yeast can be obtained starting from Y. lipolytica ST9100 which is capable of producing 24-methylenecholesterol as the dominant sterol, e.g. in an amount of at least about 20 mg/g dry cell weight when cultured at 30°C in yeast extract peptone dextrose (YPD) medium containing glucose and no sterol precursor, together with all of campesterol as the plant (24R) epimer, cholesterol, isofucosterol and desmosterol in quantifiable amount, preferably additionally with at least detectable beta-sitosterol. As indicated above, *Y. lipolytica* strain ST12178 is illustrative of such a favoured engineered *Y. lipolytica* strain, the development of which from strain ST9100 is detailed in Example 4 and table 21. This exemplifies an engineered strain of the invention which produces both 24-methylenecholesterol and campesterol as the plant epimer together with other non-native sterols favouring use in provision of a sterol mixture for feeding of honeybees. This may be by isolation of sterols from a culture medium or alternatively use of recovered yeast cells as a yeast cell biomass following culturing with subsequent drying and preferably conversion to a powder. To boost β-sitosterol it may be chosen in some instances to add a β-sitosterol supplement.

It will be appreciated that the strains construction steps set out in tables 19-21 are provided to illustrate production of specific exemplified *Y. lipolytica* strains. However, by following the same construction steps equivalent strains may be obtained starting from Y. lipolytica ST9100 or another oleaginous strain which shares all or some of the same modified genotype features compared with *Y.lipolytica* W29 strain Y-63747 as a reference strain with increased synthesis of squalene. Where use of a delta-7 reductase is specified this may be selected from any plant or animal delta-7

reductase that can be expressed in the yeast host. The delta 24(28) reductase which is expressed in strain ST12178 may be substituted by a functionally equivalent enzyme consistent with production of the 24R epimer of campesterol. Similarly the C-28 methyltransferase and delta-24(25) sterol reductase requiring steps may be carried out by providing any enzyme with the required activity. The enzymes employed may be known naturally-occurring enzymes or variants thereof which retain the required functional activity. One or more copies of each heterologous enzyme may be introduced.

Production of a sterol mixture without a sterol surrogate

Interestingly, as indicated above, it has been found that the gene for expression of the sterol surrogate can be removed from a strain of the invention as discussed above having a sequence for expression of a C-28 sterol methyltransferase with retention of useful phytosterol mixture production, including campesterol, 24-methylenecholesterol and cholesterol as the major components and including other non-native sterols in detectable amount. Such a strain has been derived from *Y. lipolytica* strain ST9100 which has been observed to produce campesterol at about 12mg/g DCW with significant 24-methylenecholesterol and cholesterol by conventional culturing in YPD medium including glucose; see discussion of engineered strain ST11441 in Example 4. Furthermore, it has been found possible by such engineering to attain a strain capable of producing a sterol mixture again including all of campesterol, 24-methylenecholesterol and cholesterol but in which 24-methylenecholesterol is the dominant sterol, i.e. present in highest quantity; see additionally the discussion of ST11542 in Example 4 and Figure 15. It will appreciated that by replacing the yeast ERG4 gene with a plant DWF1 gene, campesterol may be provided more desirably as the plant epimer for some applications.

Thus as a further aspect of the invention, there is provided use of an oleaginous yeast of the invention as discussed above which expresses a sterol surrogate and heterologous genes for production of a sterol mixture including 24-methylenecholesterol as a parent strain to produce a further yeast strain which produces a sterol mixture, where expression of the sterol surrogate is removed from said parent strain simultaneously with expression of all of a delta-7 sterol reductase, a delta 24(25) sterol reductase and a C-28 sterol methyltransferase plus attenuated ERG4 or substitute plant delta-24(28) sterol reductase (DWF1) enzyme. The resulting yeast cells may be cultured and utilised in the same way as other oleaginous yeast cells of the invention. The starting strain may preferably express all of a delta-7 sterol reductase, a delta 24(25) sterol reductase and a C-28 sterol methyltransferase plus DWF1 enzyme whereby a sterol mixture is produced comprising all of 24-methylenecholesterol, epicampesterol, cholesterol, desmosterol and isofucosterol and β-sitosterol. Removal of the sterol surrogate may be achieved with retention of production of the same sterols as illustrated by strains ST11441 and ST1152. See again strain construction tables 19 and 20.

Such a yeast-produced sterol mixture is for example of especial interest for feeding phytosterols via an artificial dietary composition to bees. Moreover, it can be envisaged that such industrially useful sterol compositions may be attained employing a wide variety of oleaginous yeasts, including *Y. lipolytica* strains without need for provision of any sterol surrogate.

Thus as an especially preferred embodiment of the invention, there is now provided an oleaginous yeast, preferably a *Y. lipolytica* strain, for producing a mixture of non-native sterols, which comprises:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5), preferably deleted ERG5;

(ii) an attenuated delta-24 sterol reductase enzyme (ERG4) or ERG4 substituted by an plant delta-24(28) sterol reductase (DWF1) enzyme providing attenuated delta-24(28) sterol reductase activity, e.g. where the ERG4 gene coding sequence or plant DWF1 enzyme coding sequence is under the control of the PrDGA1 promoter or a functionally equivalent weak promoter;

(iii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. the delta-7 sterol reductase variant of *Tetraselmis* sp. GSL018, preferably under the control of a stronger promoter than employed for (ii), e.g. the PrTEFintron promoter or a functionally equivalent promoter;

(iv) a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, e.g. the delta-24(25) sterol reductase of *S. lycopersicum*, preferably under the control of the PrGPAT promoter or a functionally equivalent weak promoter

and

(v) a heterologous nucleic acid sequence encoding a C-28 sterol methyltransferase, e.g. the C-28 sterol methyltransferase of *C. quinoa,* preferably under the control the PrGPAT promoter or a functionally equivalent promoter,

whereby a mixture of non-native sterols can be produced comprising 24-methylenecholesterol, campesterol and one or more further non-native sterols comprising cholesterol, preferably where 24-methylenecholesterol or campesterol is the dominant sterol of the mixture.

The dominant sterol, either 24-methylenecholesterol or cholesterol, may attain a production level of at least 4mg/g DCW in YPD medium including glucose and as indicated above possibly higher, e.g. at least 10 mg/g DCW. This combined with measurable cholesterol provides a sterol composition of much interest for many applications, including for example incorporation into an artificial pollen substitute for use in bee feeding.

Further modifications

The yeast of the invention described above, either including a sterol surrogate or not including a sterol surrogate, may also include a number of additional genetic modifications. These additional or further genetic modifications may be provided for improving growth or maintenance of the yeast and/or improving the content of exogenous sterol produced.

Sterol content can be increased above the levels of native sterols in wild type or reference strains. For example, the concentration of precursor may be increased. Acetyl CoA is the main precursor to the melavonate and subsequent pathways, and can be increased by overexpression of ACL (ATP citrate lyase) and/or ACS (acetyl CoA synthetase). Flux through the precursor pathways can be increased by overexpression of the biosynthetic enzymes involved, e.g. HMGR (HMG CoA reductase) and ERG12

(melavonate kinase). The activity of biosynthetic enzymes in the main sterol biosynthesis pathway can be similarly increased.

As such, yeast of the invention may include further genetic modifications in order to alter and/or improve production of endogenous sterols as described above.

For example, the yeast of the invention may further comprise reduced activity of any one or more of the following enzymes or variants thereof:

PAH1 (Mg2+-dependent phosphatidate phosphatase) which dephosphorylates phosphatidate (PA) to yield diacylglycerol. PAH1 regulates phospholipid synthesis, nuclear/ER membrane growth, lipid droplet formation, triacylglycerol synthesis, vacuolar homeostasis and cell wall integrity. PAH1 also controls transcription of phospholipid biosynthetic genes and nuclear structure by regulating the amount of membrane present at the nuclear envelope;

Bts1 (geranylgeranyl pyrophosphate synthase) which catalyses the trans-addition of the 3 molecules of IPP onto DMAPP to form geranylgeranyl pyrophosphate. Bts1 is required for membrane attachment of YPT1 and SEC4. May be involved in vesicle trafficking and protein sorting;

GDH1 (NADP-specific glutamate dehydrogenase 1) which synthesizes glutamate from ammonia and alpha-ketoglutarate;

Are1 (sterol O-acyltransferase 1) which is an endoplasmic reticulum enzyme that contributes the major sterol esterification activity in the absence of oxygen;

Say1 (steryl acetyl hydrolase 1) which is required for the deacetylation of acetylated sterols;

Sds23 (NCBI ref XP_504058.1) which is involved in DNA replication and cell separation;

Ins1 (NCBI ref XP_500057.1) which is an INSIG family protein involved in regulation of HMGR activity.

Ku70 (ATP-dependent DNA helicase II subunit 1) which is involved in non-homologous end joining (NHEJ) DNA double strand break repair and telomere maintenance; and/or

Ku80 (ATP-dependent DNA helicase 2 subunit KU80)) which is involved in non-homologous end joining (NHEJ) DNA double strand break repair and telomere maintenance.

Yeast of the invention may comprise increased or introduced activity of any one or more of the following enzymes or variants thereof:

HMG1 (3-hydroxy-3-methylglutaryl-coenzyme A reductase 1) which catalyse the conversion of HMG-CoA to mevalonate;

tHMG1 (truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1) whereby the regulatory domain is removed;

UPC2 (sterol uptake control protein 2) which is a transcription factor involved in activation of anaerobic genes such as DAN/TIR cell wall mannoprotein genes and YML083c;

Ecm22 (sterol regulatory element binding protein) which regulates transcription of sterol biosynthetic genes upon sterol depletion;

Erg1 (squalene epoxidase) which converts squalene into lanosterol;

Erg3 (sterol C-5 desaturase) which converts episterol to 5, 7, 24(28)-ergostatrienol;

Erg7 (lanosterol synthase) which converts squalene into lanosterol;

Erg8 (phosphomevalonate kinase) which is involved in the formation of farnesyl pyrophosphate;

Erg9 (squalene synthase) which converts farnesyl pyrophosphate to squalene;

Erg10 (acetyl-CoA C-acetyltransferase) which forms mevalonate from acetyl-coenzyme A;

Erg11 (lanosterol 14-α-demethylase) which is involved in the conversion of lanosterol to zymosterol;

Erg12 (mevalonate kinase) which is involved in the formation of farnesyl pyrophosphate;

Erg13 (HMG-CoA synthase) which is involved in the formation hydroxy-3-methylglutaryl-coenzyme A;

Erg19 (mevalonate pyrophosphate decarboxylase) which is involved in the formation of farnesyl pyrophosphate;

Erg20 (farnesyl pyrophosphate synthetase) which is involved in the formation of farnesyl pyrophosphate;

Erg25 (sterol C-4 methyloxydase) which is part of the C-4 demethylation complex;

Erg26 (sterol C-3 dehydrogenase) which is part of the C-4 demethylation complex;

Erg27 (sterol C-3 ketoreductase) which is part of the C-4 demethylation complex;

IDI (isopentenyl diphosphate isomerase) which is involved in the formation of farnesyl pyrophosphate;

Acl1 (ATP-citrate lyase subunit 1) which catalyses the formation of cytosolic acetyl-CoA from citrate;

Acl2 (ATP-citrate lyase subunit 2) which catalyses the formation of cytosolic acetyl-CoA from citrate;

Pot1 (3-ketoacyl-CoA thiolase) which cleaves 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA during beta-oxidation of fatty acids;

Pat1 (peroxisomal acetoacetyl-CoA thiolase) which is involved in the last step of beta-oxidation of fatty acids;

Pex10 (Peroxisomal membrane E3 ubiquitin ligase) which is required for Ubc4p-dependent Pex5p ubiquitination and peroxisomal matrix protein import;

GDH2 (NAD(+)-dependent glutamate dehydrogenase) which degrades glutamate to ammonia and alpha-ketoglutarate;

G6PD1 (Glucose-6-phosphate 1-dehydrogenase 1) which provide reducing power (NADPH) and pentose phosphates for fatty acid and nucleic acid synthesis which are involved in membrane synthesis and cell division

Are1 (sterol O-acyltransferase 1) which is an endoplasmic reticulum enzyme that contributes the major sterol esterification activity in the absence of oxygen

Are2 (acyl-CoA:sterol acyltransferase) which is an endoplasmic reticulum enzyme that contributes the major sterol esterification activity in the presence of oxygen

Atf2 (alcohol acetyltransferase) which may play a role in steroid detoxification and forms volatile esters during fermentation;

Acs1 (Acetyl-coA synthetase isoform) which catalyses the formation of acetyl-coA from acetate;

SSD1 (protein SSD1) which is a translational repressor with a role in polar growth and wall integrity;

YBP1 (YAP1-binding protein) which is involved in cellular response to oxidative stress and required for oxidation of specific cysteine residues of transcription factor Yap1p, resulting in nuclear localization of Yap1p in response to stress;

Sre1 (sterol regulatory element-binding protein 1) transcriptional activator required for transcription of genes required for adaptation to anaerobic growth like those implicated in the non-respiratory oxygen-consumptive biosynthetic pathways of sterol, heme, sphingolipid, and ubiquinone biosynthesis;

Css1 (secreted protein CSS1) which may be involved in cell wall organization and biosynthesis.

The additional modifications may be used to increase sterol esterification, lipid droplet size and/or intracellular membranes in order improve sterol accumulation in yeast of the invention.

As noted above, the *Y. lipolytica* strain ST9100 has the genotype:

MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt- >ERG12 IntC_3-SeACS<-PrGPDPrTefInt->YlACL1 IntD_1-IDI1<- PrGPD-PrTefInt->ERG20 (Arnesen et al (2020) ibid).

Oleaginous yeasts of the invention derived from ST9100 are especially preferred and will desirably retain at least all the genotype features above which contribute to increased squalene synthesis in cultured ST9100 compared to the reference *Y. lipolytica* W29 Y-63746 (ST4842) as available from the ARS culture collection such as the increased expression of HMG1, ERG12, ACL1, ACs, IDI and ERG20. It will be appreciated however that other oleaginous yeast with the same genotype may be constructed by means of yeast strain development well-known to those skilled in the field.

Methods to introduce genes

One or more of the heterologous nucleic acids encoding any of the enzymes described herein may be chromosomally integrated, or may be expressed on an extrachromosomal vector. Suitable vectors are well known. Similarly, methods of chromosomally inserting a nucleic acid are known.

For example, heterologous nucleic acids as described herein may be introduced into a yeast of the invention by methods such as heterologous recombination, site directed insertion or CRISPR-Cas9 based methods.

The heterologous nucleic acids may be inserted at any location within the yeast's genome. Preferably, the heterologous nucleic acids are inserted at one or more locations in the yeast genome that allow for relatively high expression of the heterologous nucleic acids while not having a deleterious effect on the growth of the yeast. For example, the EasyCloneYALI toolbox (Holkenbrink *et al*, 2018), defines 11 intergenic sites with high gene expression levels where integration of genes may be made.

Non-limiting examples of suitable promoters may include PrTEFintron, PrGPAT, pCyc, pAdh, pSte5, pPGK1, prGAL1, prENO2, prhp4d and prFIG1.

As indicated above, for expression of a heterologous coding sequence for non-native sterol production where strong expression is desired the yeast promoter PrTEFintron may be considered suitable or a functional equivalent thereof which can direct at least the same expression. Where weaker expression is desired, e.g. for expression of a sterol surrogate, as indicated the yeast promoter PrGPAT may be selected or a functionally equivalent weak yeast promoter. Such functionally equivalent promoters may be natural or synthetic and determined in conventional manner by fluorescent assay of GFP expression in yeast. See Holkenbrink et al (2020) *ibid*.

Nucleic acid constructs for use in providing a yeast of the invention may also comprise flanking sequences. The phrase "flanking sequence" or "homology arms" refers to a nucleic acid sequence homologous to a chromosomal sequence. A construct comprising a flanking sequence on either side of a construct (i.e., a left flanking sequence and a right flanking sequence) may homologously recombine with the homologous chromosome, thereby integrating the construct between the flanking sequences into the chromosome. Generally speaking, flanking sequences may be of variable length.

Heterologous nucleic acids provided in a yeast of the invention will generally be codon optimised. Codon optimization is a process used to improve gene expression and increase the translational efficiency of a gene of interest by accommodating codon bias of the host organism. Various codon optimization strategies have been developed by using a range of quantitative methods to generate different mRNA sequences, which can result in different levels of final protein expression. Most optimization strategies use codons with host bias to replace less frequently occurring codons.

Examples of codon optimised sequences of heterologous nucleic acids which may be included in oleaginous yeast of the invention are provided in Table 4. The sequences were codon optimised for *Y. lipolytica* using the GeneArt portal for String DNA fragments by ThermoFisher Scientific.

Nucleic acids employed in constructing yeast of the invention may include selection markers that allow for selection of yeast that include the heterologous nucleic acids of the invention. For example, markers may include antibiotic resistance.

Nucleic acids may be introduced into a yeast of the invention by any known methods, for example, by transformation methods such as lithium based methods, electroporation, biolistic methods, and glass bead methods. For example, it may be chosen to integrate heterologous sequences at intergenic loci in the *Y. lipolytica* genome as indicated above by introducing purified DNA for integration using the lithium acetate transformation protocol as described by Holkenbrink et al.(2018) Biotech J.

Production of sterol derivatives

A yeast of the invention may be used for production of one or more desired sterols or compounds derived therefrom. Thus, a yeast of the invention may additionally possess genotype features whereby it can convert one or more initially produced non-native sterols to one or more desired sterol derivatives, e g. sterol esters. Alternatively, one or more non-native sterols (or derivatives thereof) may be isolated from a yeast of the invention and subsequently converted to one or more sterol derivatives, e.g. sterol esters, for various applications. Such further conversion of initially synthesized non-native sterols may be by well-established means of common general knowledge and will be further discussed below.

Culturing and isolation of sterols from yeast of the invention

In a further aspect, the present invention provides a method for production of one or more non-native sterols which comprises culturing cells of an oleaginous yeast of the invention whereby said one or more desired non-native sterols are synthesised. Culturing may be in any suitable culture medium used for yeast. For example, the culture medium may be yeast extract peptone dextrose (YPD) media. The culture media may include a number of additional additives.

For example the culture medium may further include one or more of citrate, pyruvate, acetate, vegetable oil, glycerol, a beta-cyclodextrin to increase lipid synthesis or accumulation within the yeast, and/or increase the stability of the yeast in culture.

The culture medium may also include a carbon source. An additional carbon source may help to increase sterol production within yeast of the invention. Suitable carbon sources may include one or more of glucose, fructose, sucrose, xylose, mannose, galactose, rhamnose, arabinose, one or more fatty acids, glycerol, acetate, citrate, pyruvate, starch, glycogen, amylopectin, amylose, cellulose, cellulose acetate, cellulose nitrate, hemicellulose, xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan, lignin, lignocellulose and/or vegetable oil. Preferably, the carbon source is glucose.

Yeast of the invention may be cultured with an isotopically labelled substrate. Thus, the culture medium may include an isotopically-labelled carbon source of a type noted above, e.g. uniformly labelled 13C glucose (D-[U-13C]glucose). Use of an isotopically labelled substrate leads to production of isotopically labelled exogenous sterols. The use of isotopic labels may help with recovery of exogenous sterols from the yeast. Isotopes that may be used include ^{18}O, ^{2}H, ^{15}N and ^{13}C.

The culture medium may also include antibiotics in order to selectively allow culture of those yeast that include and express heterologous nucleic acids of the invention.

Various temperature and duration of culturing may also be used. The culturing may be performed under aerobic conditions, such as by shaking and/or stirring with aeration. The yeast may be cultured at a temperature of about 20 to about 40°C. Preferably, the yeast may be cultured at a temperature of about 30°C.

Yeast of the invention may be cultured for about 12, 24, 36, 48, 60, 72 hours or more. Preferably, the yeast may be cultivated for 72 hours or more. Yeast of the invention may be cultured for about 1 , 2, 3, 4, 5, 6, 7, 8, 9, 10 or more days.

Cultivation may preferably be by fed-batch culture, e.g. in a 5-L bioreactor, with glucose feed as the carbon source. Such fed- batch culture is well-known for yeast and may be optimised for any yeast strain of the invention to aid required sterol production as illustrated by the exemplification

Exogenous sterols produced by yeast of the invention may be stored in lipid droplets of the yeast and may be stored in esterified form.

The methods of the invention may provide one or more of the exogenous sterols at a titre of at least 0.25 mg/l. For example, the exogenous sterol may be produced by a yeast of the invention at a titre of at least 0.25 mg/l, at least 0.5 mg/ml, at least 1 mg/ml, at least 10 mg/ml, at least 20 mg/ml, at least 25 mg/ml, at least 50 mg/ml, at least 100 mg/ml, at least 150 mg/ml, at least 200 mg/ml, at least 400 mg/ml, at least 500 mg/ml, at least 600 mg/ml, at least 700 mg/ml, at least 800 mg/ml.

The methods of the invention may provide one or more of the exogenous sterols at a dry cell weight (DCW) of at least 0.01-1 mg/g DCW whereby quantification is possible but as illustrated by the exemplification a sterol mixture may include one or more sterols additionally in trace amount. For example, the exogenous sterol may be produced by a yeast of the invention at a DCW of at least 1 mg/g DCW, at least 5 mg/g DCW, at least 10 mg/g DCW, at least 15 mg/g DCW, at least 20 mg/g DCW, at least 25 mg/g DCW, at least 30 mg/g DCW, at least 35 mg/g DCW, at least 40 mg/g DCW. For example, at least 10mg/g DCW. For example, at least 25 mg/g DCW.

The methods of the invention may provide a yeast that comprises at least 20% of the one or more exogenous sterols compared to the total sterol content of the yeast, for example at least 20%, 21%, 22%, 23%, 24%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99%.

The specific exogenous sterols produced by the combinations of gene attenuations or deletions and the heterologous nucleic acids as described herein, for example as detailed in Table 2 may be the major or dominant exogenous sterol produced. That is to say that the yeast may also include other intermediate sterol compounds that may be produced during synthesis of the major sterol. The term major or dominant sterol is used to describe the main or most abundant sterol produced by a yeast of the invention. For example, the major or dominant sterol may be the sterol produced at the highest percentage of all sterols produced by the yeast i.e. highest percentage sterol of the total sterol content of the yeast. As such, yeast of the invention may produce a composition of sterols which may include one or more intermediates of the biosynthetic pathway for production of the specified sterol.

By way of example, *Y. lipolytica* strain ST12178 when grown on conventional YPD medium at 30°C with glucose at 80g/l was observed to produce 22.6 mg/g DCW of 24-methylencholesterol as the dominant sterol, 2.8.mg/g DCW campesterol as the plant epimer (24R), 0.01 mg/g DCW isofucosterol, 8.5 mg/g DCW cholesterol and 0.8mg/g DCW desmosterol plus detectable (trace amount) β-sitosterol. For this reason, it was considered of especial interest for larger scale fed-batch culture for production of a sterol mixture for various uses by incorporation into a composition, particularly an artificial dietary composition for use in artificial feeding of managed honeybees. It will be appreciated that other *Y. lipolytica* strains capable of producing the same combination of sterols (at least 24-methylenecholesterol, campesterol, cholesterol, desmosterol and isofucosterol in measurable

amount, preferably with 24- methylenecholesterol as the dominant sterol) may be obtained in accordance with the construction steps shown in table 21. As previously noted, *Y. lipolytica* ST9100 may be the chosen starting strain but an alternative starting *Y. lipolytica* strain is not excluded which shares all or some of the same modified genotype features as *Y. lipolytica* ST9100 compared with *Y. lipolytica* W29 strain Y-63746.

After culturing, in some instances the yeast may be used as a whole or the desired one or more sterols and/or derivatives may be recovered. Recovery of the sterols may be carried out by saponification which refers to the process of hydrolysis of an ester in the presence of an aqueous solution of hydroxide.

Saponified sterols may then be extracted using a hydrophobic solvent in order to provide free sterols. For example, n-hexane may be preferably employed to facilitate sterol extraction from the cell membrane. Alternatively, exogenous sterols may be recovered in esterified form.

Bi-phasic cultivation with an organic phase such as dodecane may allow for passive extraction of sterols from the cell membrane into a dodecane phase. This may reduce the toxicity of sterol accumulation and allow for greater sterol production. Sterols can be recovered from both phases.

Recovery of desired sterol(s) and /or derivatives from the culture medium simply retained within a yeast cell biomass may in some instances be preferred with subsequent inactivation. In this case, the yeast cell biomass may in some instances preferably be dried, e.g. by heating at no more than 60° C. Such heating will generally be for at least 24 hrs so that the yeast is heat-inactivated but the desired sterol(s)and /or derivatives(s) are maintained. The dried yeast cells may be used directly but more commonly will be converted into a powder for incorporation into a composition such as an artificial dietary composition, a food product, an agricultural composition, a cosmetic composition or pharmaceutical composition. Such processing to form a sterol-comprising dried powder may for example be utilised for incorporation of a sterol mixture produced by an oleaginous yeast of the invention into an artificial dietary composition for bees (see Example 5). For this purpose, the dried yeast powder may be combined with other components to provide a holidic diet at for example 20% w/w. Such a dietary composition may combine such a dried yeast powder with other components commonly considered desirable for bee development in managed honeybee colonies such as protein, lipid, sugar, minerals and vitamins. Where the sterol mixture produced in accordance with the invention is lacking β-sitosterol or has lower than desired β-sitosterol, then as indicated above additional β-sitosterol may be added.

<u>Examples of downstream products obtainable by use of yeast of the invention for sterol production</u>

The exogenous sterols, either within the yeast or recovered from the yeast may be further processed or converted to a compound derived from said sterols. For example, campesterol may be further converted to sterol drugs such as progesterone or hydrocortisone.

Phytosterols (plant sterols) such as 24-methylenecholesterol, campesterol, isofucosterol, beta-sitosterol and/or stigmasterol may be converted to brassinosteroids or withanolides.

Sterols such as cholesterol may be converted to vitamin D (cholecalciferol).

The recovered exogenous one or more sterols and /or sterol derivatives may be further incorporated into an artificial dietary composition, an agricultural composition, a food product, a cosmetic composition or a pharmaceutical composition. As indicated above, recovered cultured yeast cells of the invention may in some instances be directly incorporated into a composition, e.g. an artificial dietary composition for bees.

Artificial dietary compositions refers to a mixture of compounds that may be used for providing nutrition to a subject that are not the natural food of a subject. As indicated above, one especially favoured application of the invention is in enabling convenient production of one or more sterols or cultured yeast cells for use in feeding honeybees as a pollen substitute. One or more sterols or yeast cells may be incorporated into an artificial dietary compositions for this purpose as described in WO 2017/085477.

However, equally sterols or sterol derivatives prepared by culturing yeast cells of the invention may be incorporated into a variety of commercially useful compositions requiring one or more such components such as cosmetic compositions. Cosmetic composition refers to a non-therapeutic composition for care of the skin, hair, nails or other body parts of a subject. Sterols may be used in anti-aging creams and sun-care lotions. Oils and creams containing sterols may exhibit strong UV-protection. Cosmetic compositions may be a makeup product or a makeup-removal product. Cosmetic compositions may be serums, lotions, creams, shampoos, conditioners, oils, milks, ointments, pastes, foams, emulsions, hydrogels, shower gels, masks, lacquers, sprays or waxes.

Food compositions into which sterols prepared in accordance with the invention may be incorporated include yoghurts and milk, cheese, fat spreads, mayonnaise, salad dressing and other dairy products. It is thought that including plant stanols and sterols in the diet reduces the absorption of cholesterol. The unabsorbed cholesterol is excreted via the large bowel and this results in much less cholesterol reaching the blood supply and hence a lower blood cholesterol concentration. Total and LDL (low density lipoprotein) cholesterol levels are lowered without affecting HDL (high density lipoprotein) concentrations. Approximately 30-60% of total cholesterol is absorbed from the intestine into the blood; however when plant stanol and sterol esters are present, absorption of cholesterol may fall to approximately 20%.

One or more sterols or sterol-derived compounds obtained by culturing yeast cells of the invention may further be incorporated into a variety of pharmaceutical compositions. Sterol-derived compounds of pharmaceutical interest are well known in the pharmaceutical field, e.g. withanolides and bioactive steroidal lactones, and may be synthesized from sterols produced in accordance with the invention by known methods. Sterol derivatives can also include sterol conjugates such as sterol esters or sterol glycosides. Sterols can be conjugated to compounds such as fatty acids and sugars via the C-3 hydroxyl group.

As precursors to brassinosteroids, as previously indicated production of sterols in accordance with the invention may also find application in the agricultural field.

Similarly to sterols, other valuable compounds may be derived from squalene. Terpenoid compounds, including beta-carotene, beta-caryophyllene, beta-cryptoxanthin, lutein and linalool could be

synthesised in an oleaginous yeast optimised for increased squalene production (Arnesen et al. 2020). Synthesis of one or more of these compounds could increase the nutritional value of the oleaginous yeast when used in a food or feed. For example, a yeast strain of the invention capable of producing one or more non-native sterols could be further engineered so that it produces one or more additional desired squalene-derived compounds, for example a terpenoid compound useful as a pigment such as beta-carotene and/or a terpenoid compound useful as a scent such as linalool in an artificial dietary composition. This would increase the suitability of a yeast-based feed for insects such as bees. Such a yeast may also be cultured with subsequent recovery of one or more desired non-native sterols and/or one or more desired sterol-derived compounds and/ or one or more compounds derived from increased squalene production.

The invention is illustrated by the non-limiting exemplification provided below.

Examples

Table 1: Enzyme function and names in yeast, vertebrates and plants

Enzyme function	**Enzyme name & E.C number**		
	Yeast	**Vertebrates**	**Plants**
C-24 or C-28 sterol methyl transferase	ERG6 (2.1.1.41)		SMT1 (2.1.1.41), SMT2 (2.1.1.143)
Delta-7 sterol reductase		DHCR7 (1.3.1.21)	DWF5 (1.3.1.21)
Delta-24(25) sterol reductase		DHCR24 (1.3.1.72)	*Solanum tuberosum** SSR2 *Solanum lycopersicum* SSR2
Delta-24(28) sterol reductase	ERG4 (1.3.1.71)		DWF1 (1.3.1.72)
C-22 sterol desaturase	ERG5 (1.14.19.41)		CYP710A1 (1.14.19.41)

**Note: Solanum tuberosum* and *Solanum lycopersicum* are the only plant species known to have two delta-24 sterol reductase variants. In each case, one variant ('SSR1') is a delta-24(28) sterol reductase. This is like all other plant delta-24 reductases (plant DWF1 enzymes). The other variant ('SSR2') is a delta-24(25) sterol reductase and hence can be used as an alternative to a vertebrate delta-24 reductase (DHCR24).

Table 2: Combinations of genetic modifications for production of specific sterols:

	Product	**Gene deletion(s)**	**Heterologous gene(s)**
1	Campesterol	Δ*erg5*	Delta-7 sterol reductase
2	24-Methylenecholesterol	Δ*erg4*Δ*erg5*	Delta-7 sterol reductase
3	β-Sitosterol	Δ*erg5*	Delta-7 sterol reductase C-28 sterol methyl transferase
4	Isofucosterol	Δ*erg4*Δ*erg5*	Delta-7 sterol reductase C-28 sterol methyl transferase
5	Stigmasterol		Delta-7 sterol reductase C-28 sterol methyl transferase
6	Cholesterol	Δ*erg5*Δ*erg6*	Delta-7 sterol reductase Delta-24(25) sterol reductase
7	Desmosterol	Δ*erg6*	Delta-7 sterol reductase
8	Mixed sterol composition wherein 24-methylenecholesterol is present, preferably as the major sterol, and minor sterols may include one or more of campesterol, cholesterol, isofucosterol, desmosterol, beta-sitosterol and stigmasterol	Δ*erg5, attenuated or reduced activity of ERG4*	Delta-7 sterol reductase Delta-24(28) sterol reductase Optionally delta-24(25) sterol reductase and optionally C-28 sterol methyltransferase

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Table 3: Reference sequences of enzymes:

Enzyme	Species	GenBank Ascension No. (amino acid)	GenBank Ascension No. (mRNA)
ERG4	*Yarrowia lipolytica*	XP_503021.1	XM_503021.1
ERG5	*Yarrowia lipolytica*	XP_500188.1	XM_500188.1
ERG6	*Yarrowia lipolytica*	XP_505173.1	XM_505173.1
ERG1	*Yarrowia lipolytica*	XP_503994.1	XM_503994.1
ERG3	*Yarrowia lipolytica*	XP_503090.1	XM_503090.1
ERG7	*Yarrowia lipolytica*	XP_504990.1	XM_504990.1
ERG8	*Yarrowia lipolytica*	XP_503619.1	XM_503619.1
ERG9	*Yarrowia lipolytica*	XP_499929.1	XM_499929.1
ERG10	*Yarrowia lipolytica*	XP_500646.1	XM_500646.1
ERG11	*Yarrowial lipolytica*	XP_500518.1	XM_500518.1
ERG12	*Yarrowia_lipolytica*	XP_500956.1	XM_500956.1
ERG13	*Yarrowia_lipolytica*	XP_506052.1	XM_506052.1
ERG19	*Yarrowia_lipolytica*	XP_505041.1	XM_505041.1
ERG20	*Yarrowia lipolytica*	XP_503599.1	XM_503599.1
ERG25	*Yarrowia lipolytica*	XP_505281.1	XM_505281.1
ERG26	*Yarrowia_lipolytica*	XP_502124.1	XM_502124.1
ERG27	*Yarrowia lipolytica*	XP_501024.1	XM_501024.1
PAH1	*Yarrowia lipolytica*	BAO18767.1	AB795935.1
BTS1	*Yarrowia lipolytica*	XP_502923.1	XM_502923.1
GDH1	*Yarrowia lipolytica*	XP_505553.1	XM_505553.1
ARE1	*Yarrowia lipolytica*	XP_505086.1	XM_505086.1
SAY1	*Saccharomyces cerevisiae*	NP_011779.3	NM_001181392.3
SDS23	*Yarrowia lipolytica*	XP_504058.1	XM_504058.1
INS1	*Yarrowia lipolytica*	XP_500057.1	XM_500057.1
KU70	*Yarrowia_lipolytica*	XP_501610.1	XM_501610.1
KU80	*Yarrowia lipolytica*	XP_503443.1	XM_503443.1
HMG1	*Yarrowia lipolytica*	XP_503558.1	XM_503558.1
tHMG1	*Yarrowia_lipolytica*	XP_503558.1	XM_503558.1
ECM22	*Yarrowia lipolytica*	XP_500947.1	XM_500947.1
UPC2	*Yarrowia lipolytica*	AOW01027.1	
IDI	*Yarrowia lipolytica*	XP_504974.1	XM_504974.1
ACL1	*Yarrowia_lipolytica*	XP_504787.1	XM_504787.1
ACL2	*Yarrowia_lipolytica*	XP_503231.1	XM_503231.1
POT1	*Yarrowia_lipolytica*	XP_504109.1	XM_504109.1
PAT1	*Yarrowia_lipolytica*	XP_503808.1	XM_503808.1
PEX10	*Yarrowia lipolytica*	XP_501311.1	XM_501311.1
GDH2	*Yarrowia_lipolytica*	XP_503741.1	XM_503741.1
G6PD1	*Arabadopsis_thaliana*	NP_198428.1	NM_122970.6
ARE2	*Saccharomyces cerevisiae*	NP_014416.1	NM_001183196.1
ATF2	*Saccharomyces cerevisiae*	NP_011779.3	NM_001181392.3
ACS1	*Salmonella enterica*	WP_000083882.1	
SSD1	*Yarrowia lipolytica*	XP_505385.1	XM_505385.1
YBP1	*Saccharomyces cerevisiae*	NP_009775.3	NM_001178564.3
SRE1	*Schizosaccharomyces pombe*	NP_595694.1	NM_001021591.2
CSS1	*Saccharomyces cerevisiae*	NP_012097.1	NM_001179517.1
STC	*Tetrahymena thermophilia*	XP_001026696.2	XM_001026696.2
DHCR7 (EC 1.3.1.21)	*Solanum tuberosum*	BAQ55276.1	AB839751
	Danio rerio	NP_958487.2	NM_201330.2

	Legionella drancourtii	ACO48440.1	FJ197317.1
	Ectocarpus siliculosus	CBN77313.1	
	Candidatus Protochlamydia amoebophila	KIC71363.1	
	Coccomyxa subellipsoidea	XP_005650343.1	XM_005650286.1
	Mortierella verticillata	KFH65691.1	
	Glycine soja	XP_028244742.1	XM_028388941.1
	Tetraselmis sp.GSL018	JAC78771.1	GBEZ01006640.1
	Waddlia_chondrophila	ADI39181.1	
DHCR24 (EC 1.3.1.72)	*Danio_rerio*	AAH86711.1	BC086711.1
	Bombyx_mori	XP_004926865.1	XM_004926808.2
	Penaeus vannamei	XP_027224655.1	XM_027368854.1
	Aedes aegypti	XP_001655874.2	XM_001655824.2
	Gallus gallus	NP_001026459.1	NM_001031288.1
	Mus musculus	NP_444502.2	NM_053272.2
	Xenopus tropicalis	NP_001016800.1	NM_001016800.2
	Solanum lycopersicum	BAQ55273.1	AB83957
	Notechis scutatus	XP_026521714.1	XM_026665929.1
	Amblyraja radiata	XP_032884658.1	XM_033028767.1
SMT2 2.1.1.143	*Cucurbita pepo*	XP_023535889.1	XM_023680121
	Eutrema salsugineum SMT3	XP_006390239.1	XM_006390177.2
	Arabadopsis thaliana SMT2	NP_173458.1	NM_101884.4
	Morus notabilis	XP_010100118.1	XM_010101816.2
	Amborella trichopoda	XP_006828830.1	XM_006828767.3
	Creolimax fragrantissima		CFRG4515T1
	Ulva mutabilis		UM052_0056.1
	Rhodamnia argentea	XP_030527248.1	XM_030671388.1
	Chenopodium quinoa SMT3	XP_021737090.1	XM_021881398.1
	Glycine_soja	XP_028234656.1	XM_028378855.1
DWF1 (EC 1.3.1.72)	*Solanum_tuberosum*	BAQ55274.1	AB839749.1
	Arabidopsis thaliana	NP_850616.1	NM_180285.4
	Arachis duranensis	XP_015952627.2	XM_016097141.2
	Selaginella moellendorffii	XP_002960921.1	XM_002960875.2
	Capsicum chinense	PHU28681.1	MCIT02000001.1
	Artemisia annua	PWA66182.1	PKPP01004085.1
	Helianthus annuus	XP_022012299.1	XM_022156607.2
	Cocos nucifera	EHA8587492.1	VOII01002059.1
	Triticum urartu	EMS57493.1	KD143832.1
	Gracilariopsis chorda	PXF44537.1	NBIV01000088.1
	Capsella rubella	XP_006297345.1	XM_006297283.2
	Ajuga reptans var. atropurpurea	BAS68578.1	LC070675.1

Table 4a: Codon optimised heterologous nucleic acid sequences

Gene	**Nucleic acid sequence, codon optimised for *Yarrowia lipolytica***
Solanum tuberosum delta-7 sterol reductase	ATGGCCGAGTCTCAGCTGGTGCACCCTCCTCTGTTCACCTAC ATCTCTATGCTGGCCCTGCTGACCCTGGTGCCTCCTTTCGTG ATCCTGATGTGGTACACCAACGTGCACGCCGACGGCTCTGT GCTGCAGACCTTCAACTACCTGAAGGAAAACGGCCTGCAGG GCCTGATCGACATCTGGCCCCGACCTACCGCCATTGCCGGA AAGATCATCATCTGCTACGCCCTGTTCGAGGCTACCCTGCAG CTGCTGCTGCCCGGCAAGCGAGTGCAGGGCCCCATCTCTCC CACCGGCCACCGACCTGTGTACAAGGCCAACGGCATGGCCG CCTACACCGTGACTCTGATTACCTACCTGTCTCTGTGGTGGT TCGGCATCTTCAACCCCACCGTGGTGTACGACCACCTGGGC GAGATCCTGTCTACCCTGAACTTCGGCTCTCTGATCTTCTGC CTGTTCCTGTACATCAAGGGACACGTGGCTCCCTCTTCTACC GACCACGGCTCCTCTGGCAACATCATCGTGGACTACTACTGG GGCATGGAACTGTACCCTCGAATCGGCAAGCACTTCGACATC AAGGTGTTCACCAACTGTCGATTCGGCATGGTGTCTTGGGGA CTGCTGCCCATCACCTACTGCATCAAGCAGTACGAGGAATAC GGATCTCTGTCTGACTCCATGCTGATCCACGCCATCATCACC CTGGTCTACGTGACCAAGTTCTTCTGGTGGGAGGCCGGCTA CTGGAACACCATGGACATTGCCCACGACCGAGCCGGCTTCT ACATCTGCTGGGGCTGCCTGGTGTTCCTGCCTTGCATGTACA CTTCTCCCGGCATGTACCTGGTGAAGCACCCCGTGAACCTG GGACCTCAGCTGGCCATCTCCATCCTGGTGGCCGGCATCCT GTGCGTGTACATTAACTACGACTGCGACCGACAGCGACAAGA GTTCCGACGAACTAACGGCAAGGCCCTGGTGTGGGGCAAGG CTCCCTCCAAGATCGTGGCCTCTTACACCACCACCACTGGCG AGACTAAGTCCTCTCTGCTGCTGACCTCCGGCTGGTGGGGC CTGTCTCGACACTTCCACTACGTGCCCGAGATTCTGGCCTCT TTCTTTTGGTCTGTGCCCGCTCTGTTCAACCACATTATGCCCT ACTTCTACGTGATCTACCTGACCGGCCTGCTGCTGGACCGAG CCAAGCGAGATGACGAACGATGCAAGTCTAAGTACGGCAAGT ACTGGAAGAAGTACTGCGAGAAGGTCCCCTACCGAGTGATC CCCGGCATCTACTAA
Danio rerio delta-7 sterol reductase	ATGATGGCCTCTGACCGAGTTCGAAAGCGACACAAGGGCTCT GCTAACGGTGCTCAGACCGTTGAGAAGGAACCCTCCAAGGA GCCCGCCCAGTGGGGCCGAGCCTGGGAGGTCGATTGGTTCT CCCTGTCCGGCGTGATTCTGCTGCTGTGCTTTGCCCCTTTCC TGGTCTTCTTCTTCATCATGGCTTGTGATCAGTACCAGTGCTC TATCTCCCATCCCCTTCTGGACCTTTACAACGGTGACGCCAC TCTGTTCACCATCTGGAACCGAGCCCCCTCCTTCACCTGGGC CGCTGCCAAGATCTACGCCATCTGGGTCACCTTCCAGGTCGT TCTGTACATGTGCGTCCCCGACTTCCTGCACAAGATCCTGCC AGGTTACGTCGGCGGTGTCCAGGACGGAGCTAGAACTCCCG CCGGCCTGATCAACAAGTATGAGGTTAACGGTCTGCAGTGCT GGCTCATCACCCACGTGCTCTGGGTGCTGAATGCTCAGCACT TCCACTGGTTTTCACCCACCATTATCATTGACAACTGGATCCC CCTGCTGTGGTGCACCAACATTCTGGGCTATGCCGTCTCCAC CTTCGCTTTCATCAAGGCCTACCTGTTCCCCACCAATCCCGA GGACTGCAAGTTCACCGGAAACATGTTTTACAATTACATGATG GGTATTGAGTTCAACCCCCGAATCGGTAAGTGGTTCGACTTC AAGCTGTTCTTCAACGGTCGGCCTGGCATCGTCGCCTGGAC CCTCATCAACCTTTCCTACGCTGCTAAGCAGCAGGAGCTGTA CGGCTACGTCACCAACTCTATGATCCTGGTCAACGTCCTGCA GGCCGTGTACGTTGTCGACTTCTTCTGGAACGAGGCTTGGTA CCTGAAAACCATCGACATCTGCCACGACCACTTTGGCTGGTA CCTGGGATGGGGAGACTGCGTTTGGCTGCCTTTCCTGTACAC CCTGCAGGGTCTGTACCTGGTCTACAACCCTATCCAGCTGTC

	CACTCCCCACGCTGCCGGCGTGCTGATCCTGGGTCTGGTCG GTTACTACATTTTTCGAGTGACCAACCACCAGAAGGACCTCTT CCGACGAACTGAGGGCAACTGTTCGATCTGGGGCAAGAAGC CGACCTTTATCGAGTGCTCCTACCGATCTGCCGACGGCGCCA TCCACAAGTCCAAGCTTATGACCTCCGGCTTTTGGGGTGTTG CCCGACACATGAACTACACTGGTGACCTTATGGGTTCCCTGG CCTACTGTCTGGCCTGTGGTGGTAACCACCTCCTCCCCTACT TCTACATTGTCTACATGACTATTCTGCTGGTCCACAGATGCAT TCGAGACGAGCACCGATGCTCCAACAAGTACGGCAAGGATT GGGAACGATACACCGCCGCCGTCTCTTACCGACTGCTGCCC AACATCTTCTAA
Legionella drancourtii delta-7 sterol reductase	ATGTACTTCAAGATCCGAAACACCCTGGGACCTCTGCTGCTG ATCCTGTCTTGCCCCATCTTCGTGATGCTGATGTGGTACACC AACACCGAGCTGAAGGGATCTCTGTCTACCCTGTGGGACCTG ATCGTGCAGCAGGGCCTGTTCGAGACTACCTACAAGATCTGG CAGCCCTACTTCTGGGGCTCTGCCCTGGCCTGGAAGGTGAT CTTCGCCTTCATCATCTTCGAGCTGGCCCTGATGCGACTGCT GCCCGGCAAGGAATTCACCGGACCTGTGACTCCCAAGGGCA ACGTGCCCATCTACAAGGAAAACGGACCCCTGGCCTTCATTA CCACCATGACTACCTTTTGCGTGGCCTCTTTCGGCCTGCATC TGTTCCCCGCCTCTATCCTGTACGACAACCTGGGCGCCATCC TGGGAGCCCTGAACGTGTTCTCTCTGATCTTCTGCGCCCTGC TGTATATCAAGGGCCGATACTTCCCCTCTTCTACCGACTCTG GCATCACCAACAACATCATTTTCGACTACTACTGGGGAACCG AGCTGTACCCTCACATCTTCGGATGGTCTATCAAGAAGTTCAT CACCTGTCGATTCGGCATGATGTCTTGGGGACTGTTCCTGAT CTCTTACTGCGCCAAGCAGGCCGAGCTGGGCGACCTGGCCA ACTCTATGCTGATCTCTGTGGCCCTGCAGTTCCTGTACCTGT CTAAGTTTTACCTGTGGGAGAAGGGCTACCTGCGATCTCTGG ACATTATGCACGACCGAGCCGGCTTCTACATCTGCTGGGGCT GCCTGGTGTGGGTGCCCTGCATCTACACTTCTCCCTCTATGT ACCTGGTGCTGCACCCCATCCACCTGTCTTTCTGGCTGGCCA CCTCCATTCTGGTGCTGGGAGCCGCTTCCATCCTGATCAACT ACTTCGCCGACCGACAGCGACTGATGACCCGAGCCACCGAC GGCGAGTGTAAGATCTGGGGAAAGAAGCCCGTCACCGTTTT CGCCCAGTACCAGACCACCGAGGGCGACAAGAAGCAGACCA TCCTGCTGGCCTCTGGCTGGTGGGGCGTCGCCCGACACTTC CACTACGTGCCCGAGCTGGCTGGAACCTTCTTCTGGTCTGTG CCCGCTCTGTTTGAGAACTTCTCTCCTTACTTCTACCTGTGCT TCCTGACCATTCTGCTCGTGGACCGAGCCTTCCGAGATGACC GACGATGCTCTGACAAGTACGGACAGTACTGGCACAAGTACT GCGAGCTGGTGCCTTACAAGATCGTGCCCTTCGTGATCTAA
Ectocarpus siliculosus delta-7 sterol reductase	ATGATCGACGGCGCTGCCATCGGACGATCTCCCGTGATCTCT TCTTGGCACGGCTACAACCCCGCCTCTTCGCAGCAGCAGTGT CTGCAGGCCGTCCAGACCTCTCTGCCCACCACCGACGGCGA CCGAGAGCGACGACGATCTATGGCCCTGCGAACCTCTACCA AGCAGGCCTCTGACGCCATGGACATCCGAGAGGCCGTGAAG GCCTCTGTGGCCGCCGAGTCTAAGGACTCTAAGCTGTGGGG CTTCGTGCCCAACTGGTTCCGAACTACCGTGGGACCCCTGTT CCTGATCCTGGTGCCTCCTTTCTTCGTGGTGTTCTGGTGGCA CCTCCTGGTGTCTCACTCTGGCTCTTGGGTGTCTCTGTGGGG CGACCTGAAGGCCGCTGGACCCGAGTACGTGCTGGACGTGG TGCCCTCTCCTGTGGACCCCGCTGCCTGGAAGTACATCCTCG GCTTCGGCGTGTTCGAGATCCTGCTGATGGTGGGACTGCCC GGCAAGGCCTTCCGAGCTAACCCCACCGCCACCGGACACAT CCCCGTGTACAAGGCCAACGGCATGCTGTCTTACCTGGTGAC CCTGGCTACCCTGTGCGCCCTGGTCGCCACCGACCGACTGG ACCCCAAGAACGTGTACGACAAGCTGGGCGAGATCTTCACC GGCCTGTCTGTGTTCTCTCTGTTCTTCGTGCTGCTGCTGACC GTGAAGGGCCTGTACTTCCCCTCTACCGACGACTCTGGATCT

	AACGGATCTTTCCTGCAGAACTACTGGTGGGGCACCGAGCT GTACCCTCGAGTGTTCGGAGCCGACGTGAAGATGTTCACCAA CTGCCGATTCGGCATGATGTACTGGGCCGTGGGCGCCGTGA TCTACGCTTACACCCAGCAGCAGATGTACGGAAAGCTGTCCT CTTCTATGGCCGTGTCTGTGATCCTGCAGCTGACCTACATCA CCAAGTTCTTCCACTGGGAGATGGGCTACATGAACTCTATGG ACATTCAGCACGACCGAGCCGGCTACTACCTGTGCTGGGGC TGCCTGGTGTGGGTGCCCGCCGTGTACTCTTCTCCCGGCAT CTACCTGGTCAAGCACCCCATTTCTCTCGGCTGGTACGGCGC CTCTGCCATTCTGGCCCTGGGCCTGCTGTCTATCTGGGCCAA CTTCGACGCTGACCGACAGCGACACGCCTTCCGACAGGCCA AGGGCGACATCATCGTGTGGGGCAAGCCCGCCAAGTACATC ACCGCCGGCTACATCAACGCCCGAGGCGAGAAGGCCTCCTC TCTGCTGCTCTGCACCGGCTGGTGGGGAGTCGCCCGACACT TCCACTACCTGCCTGAGATTACCGGCGCCTTCTTCTGGACCG TGCCTGCTCTGTTCGAGACTCCCACTCCTTACTTCTACGTCGT GTTCCTGGTGCTGCTCCTCACTGACCGAGCTTTCCGAGATGA CACCCGATGCCGAGGCAAGTACGGCAAGCACTGGGACAAGT ACTGCGCTCAGGTGCCCTACAAGATCGTGCCCGGCATCCTG TAA
Candidatus *Protochlamydia amoebophila* delta-7 sterol reductase	ATGCTGATCGAGATGCTGTGCATCACCAAGCACATCCTCGAG AAGATCTCTAAGTTCTCTCTGGCTCGACCCCACGTGGCCAAC ACCAACATTTTCTTCAGACAGACCTTCGGACCCCTGTTCCTG CTGCTGCTGTGCCCTCCTACCGTGTTCGCCTTCTGGTACACC AACACCTACCTCGAGGGATCTCTGTTCCGATTCACCGAGTTC GCCTGGCAGCAGGGCTTCCTGTCTACCCTCAAGACTATCTGG TTCCCCTACTTCTTCGGCACCTCTATCGCCTGGACCATGCTG GCCATCTTCGCTTCTCTGCAGCTGATCCTGATGCGAATTCTG CCCGGCGAGTGCTACGAGGGCCCCATCACTCCCACCGGACA CGTGCCCCTGTACAAGGCCAACCGATTCTCTGCCTTCATCGT GACCGTGTCTATCTTCCTGATCGCCTCTTGCTACTACCAGCT GTTCGCTCCCACCATCATCTACGACAACTTCCCCGGCCTGCT GGGCGCCCTGAACATCTTCTCCCTGTGTTTCTGCTTCTTCCT GGTGCTGAAGGGCCACTACTTCCCTTCTAACGGCGACGTCG GCGGCTCTGGCAACATCATCTTCGACTACTACTGGGGCATGG AACTGTACCCTCGACTGCTCGGCTGGGACATCAAGCAGTTCA CCAACTGCCGATTCGGCATGATGTCTTGGGCCCTGATCGTGA TCTCTTTCGCCGCCAAGCAGCAACAGCTGGACGGCCTGTCT GACTCTATGTTCGTGGCCGTGGCTCTCCAGCTCATCTACATT ACCAAGTTCTTCATCTGGGAGCCCGGCTACCTGCGATCTCTG GACATTATGCACGACCGAGCCGGCTACTACATCTGCTGGGG CTGCCTGGTGTGGGTGCCCGGCATCTACACTTCTCCCACTCT GTACCTGGTGGATCACCCCAACCACCTGGGACTCGCCCTGT CCTCTCTGCTGTTCGTGGTGGGCGTGATCGGCATCCTGGTG AACTACCTGGCCGACCGACAGCGACAGCTCGTGCGAAAGAA CCAGGGCAACTGTCGAATCTGGGGAAAGGAACCCATCCTGA CCATTGCCAAGTACACCACTCAGACCGGCGAGACTAAGCAGA ACCTGCTGCTCGCCTCTGGCTGGTGGGGCCTGTCTCGACAC TTCCACTACCTGCCTGAGCTGCTGGGAGCCTTCTGCTGGTCT GCTCCCGCTCTGTTCGAGAACTTCCTGCCTTACTTCTACTTCG TGTTTCTGACCCTGCTCCTGACCGACCGAGCTTTCCGAGATG ACCAGAGATGCTCTAAGAAGTACGGCGAGGACTGGAAGATCT ACTGCCAGCGAGTGCCCTACAAGATCATCCCCTTCGTGATCT AA
Coccomyxa subellipsoidea delta-7 sterol reductase	ATGGTCACAACCCGAGCCGCTGCTCGAGCTCAGACCCCTCT GGGCAAAGCTCCTCCCTCCGACCTGTCCCACTCCGAACCCT CCGTCTCTACTCAGAACGGCAAGAAGACATGGGCCGAGACC GCTGGGGCAGGCGAGCACATCGGCGCCTGGGGTATCGGCG GCACTGCCGGACACGTCTTGGCCTACCTGGGTACTCTTGCC CTTATGATCGGTTGCCCCGCCTTTGCAATCTACATGTGGTTTA

	CCCTCACCCACTTGGATGGCTCTCTTGTGGAGCTCGTCCAGT TCGCCCAGAAGGCTGGATTTCAGGGCGTCCGAGCCTCATGG CCCTGGCCCTCCCAGGAGGCCTGGGCCATCATCGCCTCCTT CGGCGGTCTTCAGGCCTTCCTGCAGCTTGCCTTGCCCGGTG CTGTGCACAAGGGCCCAGTCTCTCCCAAGGGTAACGTCCCC GTGTACAAGGCCAACGGTGTCCTCGCCTACTTTACCACCCTG GCTCTGTTCGTCCTTGGCTGGCAGTTCAAGCTGTTTTCCCCC GCCAGAGTCTACGACCTGTTCGGCGAGATCCTTTCCGGCCT GAACATGTTCTCTCTGCTGTTCTGCCTGTTCCTGTACTTCAAG GGCAAGTACGCCCCCTCATCCTCGGATTCTGGTTCTACCGGC TCCCTGATGTACGATTATTACTGGGGGATGGAGCTGTACCCT CGGATTGGACGACACTTTGACCTGAAGACTTGGACTAACTGC CGAATGGGCATGATGGGCTGGGGAGTTCTGGTCCTGTGTTA CGCTGTGAAGCAGCACGAGCTGTACGGTTACCTTTCTAATTC GATGGCTGTTTCTATCCTGCTCATGCATTTATACATTTTCAAG TTCTTCCTCTGGGAGACTGGTTACTGGGGTACCATGGACATC GCTCACGACCGCGCTGGATACTACCTCTGCTGGGGATGCCT GAACTGGGTCCCCGCTATCTACACCTCTCCCGCCCTGTACCT CGTCGAGAACCCTATTCAGTGGTCTCTGCCCGCCGCCACCG CTATCGCTGTTGCCGGTACCCTGGCTATCTACATCAACTACG ACTCCGATCGGCAGCGACAGGTTTTCCGAGCTACCAATGGTA AGGCCCTGGTGTGGGGCAAGCCTCCCCAAATTATCTCTGCCA AGTACATCACCGGCGATGGCAAGCAGAAGACCTCCCTGCTC CTGGCCTCTGGCTGGTGGGGGCTCGCCCGACACTTCCACTA CCTGCCCGAGATCTTGGCCGCCTTCTTTTGGACCCTCCCCGC TGGTATCTCCCACGCTCTGCCCTACTTTTACGTCTTCTTCCTG ACTCTGCTCCTTACCGATCGAGCTTTCCGAGACGACGTCCGA TGTAGCTCCAAATACGGTGCCTACTGGCAGCAGTACACCAAG GCCGTTCCCTACAAGATGATCCCTTACATTTTCTAA
Mortierella verticillata delta-7 sterol reductase	ATGGCCGTGCAGCAGCGAAAGACCCCTGCTCAGGTGGACGT GAAGGCCGAGTCTAAGCTGGACGCCCAGGTGGGCAAGACCT GGGGCCGAGATCGAGATGTGTCTTTCGGCACCATCCTGATCT CCCTGGGCATCCTGGTGATGTCTCCCATCTGGGTGATGTACA CCTACATCTCTTGCAACGCCTACCAGTGCGCCATGTCTGCTC CCGCTCTCGAGATCTACAACTCTCCCGACACTCTGGCCGCCA TTCAGACCCTGCTGCTGCGAGAGGTGCCCCGATTCTCTCCCT ACGCCGCTCGACTGTTCTTCACCTGGCTGGCCTTCCAGGCC GCTCTGTACGCCTTCCTGCCTGCTCAGATCGGCTACGGCCA GCGAACCCCTGCCGGCCACATTCTGCCCTACAAGGTGAACG GCCTGCTGGCCTGGTTCATCTCTCACTCTATCTACGCTGCCG GCGGACTGTACTTCGGCTGGTGGAAGCTGTCTATCATCCACG ACAACTGGGGCGGACTGCTGGTGGCCGCCAACATGTACGGC TACTTTCTGACATTCTTCTGCTTTATCAAGGCCTACACCTTTC CTTCTCACCCCGCCGACCGAAAGTTCTCTGGCTCTTTCATCT ACGACCTGCTGATGGGCATCGAGTTCAACCCTCGAATCGGCA AGCTGTTCGACTTCAAGCTGTTTCACAACGGACGACCCGGCA TCGTGGCCTGGACCATGATCAACCTGTCCTTCGCCGCTGCTC AGTACGAGAAGATCGGATACGTGACCAACTCTATGATCCTGC TGAACCTGCTGCATGCTACCTACGTGCTGGACTTCTTCTACA ACGAGGACTGGTATCTGCGAACCATCGACATTGCCCACGACC ACTTCGGCTTCTACCTGGCCTGGGGCGACTCCGTGTGGCTG CCCTGGCTGTACACCCTGCAGTCTCACTACCTGGTGCGAAAC CCCGTGGACCTGACTCCTGTGCAGTTCGCCTTCGTGTTCACC GTGGGCTACATCGGCTACTTCATCTTCCGATCTGTGAACCAC CAGAAGGACATCGTCCGATCTACCAACGGCGAGTGCATGATC TGGGGCAAGCCCGCCAAGGTGATCCGAACCTCTTTCGTGAC CTCTGACGGCAAGACCCACAAGTCTCTGCTGCTGTGCTCTGG CTACTGGGGCCTGTCTCGACACTTCAACTACGTGGGCGACCT GCTCATCTCTCTGGCCATGTGCATGACCTGCGGCACCCAGCA TCTGCTGCCCTACTTCTACATCATCTACATGACCATCCTGCTG

	CTCCACCGAATCCAGCGAGATCACACCCGATGCAAGGGCAA GTACGGAAAGTACTGGGACGAGTACATGAAGGCCGTGCCTT ACAAGCTGATCCCCTACGTGTACTAA
Glycine soja delta-7 sterol reductase	ATGGGCGCTACCGTGCACTCTCCCCTGGTGACCTACGCCTCT GTGATCTCTCTGCTGACCCTGTGTCCTCCTTTCGTGGTGCTG CTGTGGTACACCATGACTCTGGCCGACGGCTCTGTGTCTGAG ACTTTCCACTACCTGCGACAGAACGGCCTGCAGGGCCTGCT GCACATCTGGCCCACTCCTACTCCTACCGCCTGCAAGATCAT TGCCGTGTACGCCGCCTTCGAGGCCGCTCTGCAGCTGCTGC TGCCCGGCAAGACCGTGTACGGCCCCATCTCTCCCACCGGC CACCGACCTGTGTACAAGGCCAACGGACTGCAGGCCTACTT CGTGACCCTGATCACCTACTTCGCCCTGTGGTGGTTCGGCAT CTTCAACCCCACCATCGTGTACCACCACCTGGGCGAGATCTA CTCTGCCCTGATCTTCGGCTCTTTCCTGTTCTGCGTGTTCCTG TACATCAAGGGCCATCTGGCTCCCTCTTCTACCGACTCTGGA TCTTCTGGCAACCTGATCATCGACTTCTACTGGGGCATGGAA CTGTACCCTCGAATCGGCAAGCACTTCGACATCAAGGTGTTC ACCAACTGTCGATTCGGCATGATGTCTTGGGCCGTGCTGGCC CTGACCTACTGCATCAAGCAGTACGAAGAGAACGGCAAGGT GGCCGACTCTATGCTGGTCAACACCGCTCTGATGCTGGTGTA CGTGACCAAGTTCTTCTGGTGGGAGGCCGGCTACTGGTCTA CCATGGACATTGCCCACGACCGAGCCGGCTTCTACATCTGCT GGGGCTGCCTGGTGTGGGTGCCCTCTGTGTACACCTCTCCT GGCATGTACCTGGTGAACCATCCTGTGAACCTGGGCATCAAG CTGGCTCTGTCTATCCTGGTGGCCGGCATCCTGTGCATCTAC ATCAACTACGACTGCGACCGACAGCGACAAGAGTTCCGACG AACTAACGGAAAGGGCACCGTGTGGGGCAAGGCCCCTTCTA AGATCGAGGCCACCTACACCACTACCTCTGGCGAGACTAAGC GATCCCTGCTGCTGACCTCTGGCTGGTGGGGCCTGTCTCGA CACTTCCACTACGTGCCCGAGATCCTGGCCGCCTTCTTCTGG ACCGTGCCTGCTCTGTTCGAGCACTTCCTGCCTTACTTCTAC GTGATCTTCCTGACCATCCTGCTGTTCGACCGAGCTAAGCGA GATGACGACCGATGCCGATCTAAGTACGGCAAGTACTGGAA GCTGTACTGCGACAAGGTGCCCTACCGAATCATCCCCGGCAT CTACTAA
Tetraselmis sp. GSL018 delta-7 sterol reductase	ATGAAGCGAGCCTCCAAGACCCCCGACACCGCCTCTAAGGG TCGAGAACCCCTTTCTGAGCCTCACACCAACGGTGTCGCTAA GGCCAGCAACAAGACCTCTTGGGCCGAGTCCAATGGCATCG GTGATCGAGACGGATTCATGGGCCTGTCCGGTGCCGCCGGC CATGCTGTGGCCCTTCTGGGCACCGTCGTGCTGCTCGTCGG TTGCCCCGCCTTTGTCTTCGTGCTCTGGTACATTAATTGTCGA CTCGACGGCTCCGTCTCCGAGTTCGTCGCCCTCGCCGCTCG AGAGGGCGCCGTGGGTCTCTGGCAACGATGGCCTACTCCCA CTGCTGAGGCCTGGGCCATCATTGGCACCTTCGGTGCAGTC GAGGCCTTCCTGCAGCTGGCTCTCCCTGGCAAGAAGTTCCT GGGTCCCGTCTCTCCTAAGGGTAACGTCCCCGTCTACAAGG CCAACGGCATGCAAGCCTACGTGACTACCCTGGTCCTGTTCT TTGCCGTCTGGGGCTCCGGCATCTACAACCCCGCGCGAGTC TACGATCTCATGGGTGAGATTCTGGCCGCCCTGAACATCTTT TCTCTGCTGTTTTGCCTGTTCCTCAACATTAAGGGTCATGTCG CCCCTTCCTCTACTGACTCCGGCTCTACCGGCTCTTTGCTGT ACGACTACTACTGGGGCATGGAGCTTTACCCTAGAATTGGCC GATCCTTCGACATTAAGACCTGGACCAACTGCCGAGTCGGCA TGATGGGTTGGGGCATCCTGATCCTGTGCTACGCCGCCAAG CAGGTGGAGGAGGCTGGATTTCTGTCCGACTCCATGGCTGT GTCCGTCATTCTCATGCACGTTTACATCGCCAAGTTCTTCTGG TGGGAGACTGGTTACTGGAAGACCATGGACATCATGCACGAT CGAGCCGGTTACTACATCTGCTGGGGTTGCCTCGTGTGGATC CCCTCCATGTACACCTCTCCCACCATGTTTCTTGTCAAGCATC CTATGGTGCTGGGCCCCACCCTCACCGGCGCTGTCCTGGCT

	GCTGGCCTGCTGTGTATCTACATCAACTACGACGCCGACCGA CAGCGACAGGTTTTCCGAGAGTCTAACGGCAAGGCCCTGATT TGGGGTCGAAAGCCTAAGAAGATCGAGGCCCAGTACACTAC CGCGGACGGCCAAACTAAGACCTCTCTGCTGCTGGTGTCTG GTTGGTGGGGTGTCTCCCGACATTTCCACTACCTGCCCGAGA TACTTGCCTCCGTGTTCTGGTCTGTGCCCGCCCAGACTGATT ACGCTATGGCCTACCTGTACTCTGCCTACCTCACCATTCTTCT CGTGGACCGTGCCTTCCGAGACGACCTGCGATGCGCTTCCA AGTACGGCAAGCACTGGGTCGAGTATTGCCGACAGGTCCCC CACAAGATCGTGCCCTACATCTTCTAA
Waddlia chondrophila delta-7 sterol reductase	ATGGCCGCCACCACCACCAACGTGCAGACCCGAAACTGGGG CCGAGCCTGGGAGACTACCTGGCTGTCTCTGTTCTCTACCAT TGCTCTGCTGGCCACCGCTCCTATGATGGTGCTGTACTGCTA CATTGCCTGCGTGCGATTCCGAGGCTCTCTGATCGGACCCG CCTACGCTCTGGCCTCTGGCGCCGTGTCTCTGGACTCTCTGT TCCCCTCGTTCGAGGTGGGCATCTTCGCCCTGTACCTCGGCT GGTTCGCCTTCCAGCTGCTGCTGTACCTGGGACTGCCCGAC CTGCTGCACCGAATTCTGCCCCGATACCGAGGCGGCCGACA AGAGGGCGCTGTGACCCCTGCCGGCAAGCAGCTGGTGTACC AGATCAACGGACTGCAGGCCTGGCTGATCTCCCACCTGTTCT TCGGCATCGGCGCCTACGTGCTCGGATGGTTCTCTCCCTCG ATCATTGCCGAGAACTGGGGAGGCTTCCTGATCGTGACCAAC GTGATGGGCTACCTGACCGCCATCTTCGTGTACGTGAAGGC CTACCGATTTCCTTCGAACGCCGAGGACCGAAAGTTCTCTGG CAACCCTCTGTACGACTTCTTCATGGGCATCGAGTTCAACCC TCGAATCGGCAAGTTCGACTTCAAGCTGTTCTTCAACGGACG ACCCGGCATCATTGCTTGGACCCTGATCAACTGGTCTTTCGC CGCCAAGCAGTACGCCGACCTGGGATACCTGCCTAACTCTAT GCTGCTGGTGAACGTGCTGCAGGCTATCTACGTGCTGGACTT CTTCTGGCACGAGACTTGGTATCTCAAGACCATCGACATCTG CCACGACCACTTCGGCTGGATGCTGTCTTGGGGCGACCTGG TGTGGCTGCCCTACATGTACACCCTCCAGGGCCTGTACCTGC TCTACCATCCTGTGGACCTGTCTACCGGCTTCGCTCTGTTCG TGCTGACCCTGGGCGTCGTGGGCTACGCCATTTTCCGATCTG CCAACCACCAGAAGGACCACTTCCGACGAGTGCAAGGCAAG GAACCCATCTGGGGAAAGATGCCCGAGTTCATCTCTTGCCAG TATACCGCCGCTGACGGCTCTCTGCACCACACCAAGCTGCTC CTCTCCGGCTGGTGGGGACGAGCCCGACACATGAACTACAC CGGCGACCTGATGCTGTCCCTGGCCTACTGCCTGGCCTGCG GCTTCTCTCACCTCCTGCCTTACTTCTACTTCGTCTACATGAC CATCCTGCTGGTCAACCGATGCTACCGAGATGAGCACCGATG CGAGAACAAGTACGGCGACGCCTGGCGAAAGTACTGCCGAC GAGTCCCCTACCGACTGATCCCCGGCATCTACTAA
Tetrahymena thermophilia squalene-tetrahymanol cyclase	ATGAAGAAGATCCTCATCGGTCTCATCATCGGTCTCTTCCTCT TCTCCTCCGTCAACGCCTCCGTCAACCTCACCGAGGTCCAGA ACGCCATCTCCATCCAGCAGGGTATCAACTGGGCTGAGGTC CACAACAACACCTGGTACTACCCTCCCTACCTCGGTGAGATG TTCATCTCCGAGTACTACTTCGAGCTCCTCGTCCTCAACTGG ACCCACAAGTCCGCCTTCAACGCCACCTACTTCACCGAGCGA CTCCTCCAGACCCAGTTCGAGGACGGTTCCTGGGAGCAGGT CCGAGAGCAGAACCTCGAGACCGGTCAGCTCGACGCCACCG TCTTCAACTACTGGTACCTCAAGTCCATCAACAACAACCCCAA GATCGAGGCTGCCCTCCAGAAGGCCCGAAAGTGGATCGTCG CTCAGGGTGGTATCGAGGCCACCCAGACCATGACCAAGTTC AAGCTCGCTGCCTTCGGTCAGTACTCCTGGGAGGACCTCTG GTACGTCCCTCTCTTCATCTTCAAGCAGAACGGTATCTTCAAG TACACCTACGTCAAGGACATCGTCGCCCAGTGGGTCTACCCC CACCTCACCGCTCTCGCCTACCTCCGATACCAGCGAACCGTC TTCAACGTCCCTGTCGCTGACCTCCGAGAGCTCTGGATCAAC TACCCCAAGAACGGTATCAAGATCTCCCCCCGAGAGTACTCC

	ACCCTCAACCCCGACTCCGACCTCCTCATCCTCATGGACGAG ATCTTCAAGCTCAAGCAGCCTCTCGGTTCCTTCGGTGCCTAC ACCATCTCCACCCTCCTCACCCTCATGTCCTTCAAGGACTTC CAGTCCAAGCACCCCCACCTCTACCAGAACGAGATCCAGAAG GCCTACGAGGACGGTTACTACTTCGTCGAGTTCAACTACTTC AACTTCCGAGAGGCCTACCACGGTTCCCTCGACGACGGTCG ATGGTGGGACACCATCCTCATCTCCTGGGCCATGCTCGAGTC CGGTCAGGACAAGGAGCGAATCTTCCCCATCGTCCAGAACAT GGTCAAGGAGGGTCTCCAGCCCAAGAAGGGTATCGGTTACG GTTACGACTTCGAGTACGCTCCCGACACCGACGACACCGGT CTCCTTCTCGTCGTCATGTCCTACTACAAGGAGGCCTTCCAG AAGCAGATCCCCGAGACCATCGAGTGGCTCTTCTCCATGCAG AACGACGACGGTGGTTACCCCGCCTTCGACAAGGGTAAGAA CGAGGACAACCTCCTCTTCAAGTTCGCCTTCAACATGGCCGG TATCGCCAACTCCGCCGAGATCTTCGACCCTTCCTGCCCTGA CATCACCGGTCACATCATGGAGGGTCTCGGTGAGTTCGGTTA CCAGGCCAACCACCCCCAGATCCAGAACATGATCAAGTACCA GCGAAAGACCCAGAACAAGTGGGGTTCCTGGCAGGCTCGAT GGGGTGTCAACTACATCATGGCTGTCGGTGCTGTCGTTCCTG GTCTCGCTCGAGTCAACTACGACCTCAACGAGCAGTGGGTC CAGAACTCCATCAACTACCTCCTCAACAAGCAGAACAAGGAT GGCGGCTTCGGTGAGTGCGTCCTCTCCTACAACGACCCCGA GAAGTGGAACGGTATCGGTAAGTCCACCGTCACCCAGACCT CCTGGGGTCTCCTCGCTCTCCTCGAGGTCTACAACCAGAACG AGCAGATCAAGCACGCTGCCGATCGAGCTGCCCAGTACCTC CTCGACCAGTTCAAGCGAGACGACAACACCTTCTACGACCAC TCCACCATCGGTACCGGTCACCGAGGTCTCCTCTACCTCCAG TACCCCTCCTACGCCCAGTCCTTCCCTCTCGTCGCCCTCAAC CGATACCAGAAGATCTCCCAGGGTCAGTACCACTTCTCCAAG AACCTCTACAACGGTAACGGTGAGCCCGTCCAGAAGCAGAA CATCTAA
Salmonella enterica acetyl-CoA synthetase	ATGTCTCAGACCCACAAGCACGCTATCCCCGCCAACATTGCC GACCGATGCCTGATCAACCCCGAGCAGTACGAGACTAAGTAC AAGCAGTCTATCAACGACCCCGACACCTTCTGGGGCGAGCA GGGCAAGATCCTGGACTGGATCACCCCTTACCAGAAGGTCAA GAACACCTCTTTCGCTCCCGGCAACGTGTCTATCAAGTGGTA CGAGGACGGCACCCTGAACCTGGCCGCCAACTGCCTGGACC GACACCTCCAAGAGAACGGCGACCGAACCGCCATCATCTGG GAGGGCGACGACACCTCTCAGTCTAAGCACATCTCTTACCGA GAGCTGCACCGAGATGTGTGCCGATTCGCTAACACCCTGCT GGACCTGGGCATCAAGAAGGGCGACGTCGTCGCTATCTACA TGCCCATGGTGCCCGAGGCCGCCGTGGCCATGCTGGCCTGC GCTCGAATCGGCGCCGTGCACTCTGTGATCTTCGGCGGCTT CTCGCCCGAGGCTGTGGCCGGACGAATCATCGACTCCTCTT CTCGACTGGTGATTACCGCCGACGAGGGCGTGCGAGCCGGC CGATCTATTCCCCTGAAGAAGAACGTCGACGACGCTCTGAAG AACCCCAACGTGACCTCTGTCGAGCACGTGATCGTGCTGAAG CGAACCGGCTCTGACATCGACTGGCAAGAGGGCCGAGATCT GTGGTGGCGAGATCTGATCGAGAAGGCTTCTCCCGAGCACC AGCCTGAGGCCATGAACGCTGAGGACCCTCTGTTCATCCTGT ACACCTCTGGCTCTACCGGCAAGCCCAAGGGCGTGCTGCAC ACCACCGGCGGCTACCTGGTGTACGCCGCCACCACCTTCAA GTACGTGTTCGACTACCATCCTGGCGACATCTACTGGTGCAC CGCTGACGTCGGCTGGGTGACCGGCCACTCTTACCTGCTGT ACGGACCCCTGGCCTGTGGCGCTACCACTCTGATGTTCGAG GGCGTCCCCAACTGGCCCACTCCTGCTCGAATGTGCCAGGT GGTGGACAAGCACCAGGTGAACATTCTGTACACCGCTCCTAC CGCCATTCGAGCCCTGATGGCCGAGGGCGACAAGGCCATCG AGGGCACCGACCGATCTTCTCTGCGAATCCTGGGCTCTGTG GGCGAGCCTATTAACCCCGAGGCCTGGGAGTGGTACTGGAA

	GAAGATTGGCAAGGAAAAGTGCCCCGTCGTTGACACCTGGT GGCAGACCGAGACTGGCGGCTTCATGATTACCCCTCTGCCT GGCGCCATCGAGCTGAAGGCCGGCTCTGCTACCCGACCATT CTTCGGCGTGCAGCCCGCTCTGGTCGACAACGAGGGACACC CTCAAGAGGGCGCCACCGAGGGCAACCTGGTCATCACCGAC TCTTGGCCCGGACAGGCTCGAACCCTGTTCGGCGACCACGA GCGATTTGAGCAGACCTACTTCTCTACCTTCAAGAACATGTAC TTCTCTGGCGACGGCGCTCGACGAGATGAGGACGGCTACTA CTGGATTACCGGCCGAGTGGACGACGTGCTGAACGTGTCTG GCCACCGACTGGGCACCGCCGAGATCGAGTCTGCCCTGGTG GCTCACCCCAAGATCGCCGAGGCTGCCGTCGTGGGCATTCC CCACGCCATCAAGGGCCAAGCCATCTACGCCTACGTGACCC TCAACCACGGCGAGGAACCCTCGCCTGAGCTGTACGCCGAG GTGCGAAACTGGGTGCGAAAGGAAATCGGTCCCCTGGCTAC CCCTGACGTCCTGCATTGGACCGACTCGCTGCCCAAGACAC GATCTGGAAAGATCATGCGACGAATCCTGCGAAAGATCGCTG CCGGTGACACCTCTAACCTGGGCGACACTTCTACCCTGGCTG ACCCTGGCGTGGTCGAGAAGCCCCTGGAAGAGAAGCAGGCC ATTGCTATGCCCTCTTAA
Solanum lycopersicum delta-24 sterol reductase	ATGTCTGACGCCAAGGCTCCCGTGGCCACTGCTTACCCCAA GCGAAAGATCCAGCTGGTGGACTTCCTGCTGTCTTTCCGATG GATCATCGTGATTTTCTTCGTGCTGCCCTTCTCTTTCCTGTAC TACTTCTCTATCTACCTGGGCGACGTGAAGTCTGAGCGAAAG TCTTACAAGCAGCGACAGATGGAACACGACGAGAACGTGAA GGAAGTGGTGAAGCGACTGGGCCAGCGAAACGCCGAGAAG GACGGCCTGGTGTGCACCGCTCGACCTCCATGGGTCGTCGT GGGCATGCGAAACGTGGACTACAAGCGAGCCCGACACTTCG AGGTGGACCTGTCTAAGTTCCGAAACATCCTGGACATCGACA CCGAGCGAATGGTGGCCAAGGTCGAGCCCCTGGTGAACATG GGCCAGATGTCTCGAGTGACCATTCCTATGAACCTGTCTCTG GCCGTGCTGGCCGAGCTGGACGACCTGACCGTCGGCGGCC TGATCAACGGCTTCGGCGTCGAGGGATCTTCTCACATCTTCG GCCTGTTCTCTGACACCGTGGTGGCCCTCGAGGTGGTGCTG GCTGACGGCAAGGTGGTGCGAGCCACCAAGGACAACGAGTA CTCTGACCTGTTCTACGCTATCCCCTGGTCGCAGGGCACCCT GGGCCTGCTGGTGTCTGCCGAGATCAAGCTGATCCCCGTGG ACCAGTACGTGAAGCTGACCTACAAGCCCGTGCGAGGCAAC CTGAAGGAACTGGCCCAGGCCTACGCCGACTCTTTCGCTCC CAAGGACGGCGACCAGGACAACCCCTCTAAGGTGCCCGAGA TGGTCGAGGGCATGATCTACGGCCCCACCGAGGGCGTGATG ATGACCGGCATGTACGCCTCTCGAAACGAGGCCAAGCGACG AGGCAACGTGATCAACAACTACGGCTGGTGGTTCAAGCCCTG GTTCTACCAGCACGCTCAGACCGCTCTGAAGCGAGGCGAGT TCGTCGAGTACATCCCTACTCGAGACTACTACCACCGACACA CCCGATCTCTGTACTGGGAGGGCAAGCTGATTCTGCCCTTCG GTGACCAGTTCTGGTTCCGATTCCTGCTCGGCTGGCTGATGC CTCCTAAGATCGCCCTGCTGAAGGCTACCCAGTCTGAGGCCA TCCGAAACTACTATCACGACCACCACGTGATCCAGGACCTGC TCGTGCCCCTGTACAAGGTGGGCGACTGCCTCGAGTGGGTG CACCGAGAGATGGAAGTGTACCCCATCTGGCTGTGTCCCCA CCGAATCTACAAGCTGCCTGTGCGACCTATGATCTACCCCGA GCCTGGCTTCGAGAAGCACAAGCGACAGGGTGACACCGAGT ACGCCCAGATGTACACCGACGTGGGCGTGTACTACGTGCCC GGTGCCGTGCTGCGAGGTGAGCCCTTCGACGGCTCTGAGAA GTGCCGACAGCTGGAACTGTGGCTGATCGAGAACCACGGCT TCCAGGCTCAGTACGCCGTGACCGAGCTGACCGAGAAGAAC TTCTGGCGAATGTTCGACAACGGCCTGTACGAGCAGTGCCG ACGAAAGTACAAGGCCATCGGCACCTTCATGTCTGTGTACTA CAAGTCTAAGAAGGGCCGAAAGACTGAGAAGGAAGTTCAAGA

	GGCCGAGCAAGAGAAGGCTGAGCAAGAAACCCCTGAGGCCA ACTAA
Chenopodium quinoa C-28 sterol methyltransferase	ATGGACTCTATGGCCCTGATCTGCACCGTGGGCCTGCTGTTC GGCGGCCTGTACTGGTTCATCTGCATTCACGGACCCGCCGA GCGAAAGGGCAAGCGAGCCGTGGACCTGTCTGGCGGCTCTA TCTCTTCTGACAAGGTGCAGGACAAGTACCAGCAGTACTGGT CGTTCTTCCGACGACCTAAGGAAATCGAGACTGCCGAGAAG GTGCCCGACTTCGTGGACACCTTCTACAACCTGGTGACCGAC ATCTACGAGTGGGGCTGGGGCCAGTCTTTCCACTTCTCTCCC TCGATTCCCGGCAAGTCTCACCGAGATGCTACCCGAATCCAC GAAGAGATGGCCGTCGACCTGATCAAGGTGTCTCCCGGCCA AAAGATCCTGGACGTCGGCTGCGGCGTCGGCGGACCCATGC GAGCCATTGCCGCTCACTCTCGAGCCAAGGTGACCGGCATC ACCATCAACGAGTACCAGGTGAAGCGAGCCAAGCTGCACAA CAAGAAGGCCGGACTGGACTCTCTGTGCGAGGTGGTGTGCG GCAACTTCCTCGAGATGCCCTTCGCCTCTAACACCTTCGACG GCGCCTACTCTATCGAGGCCACCTGTCACGCTCCCAAGCTG GACGACGTGTACTCTGAGATCTTCCGAGTGCTGAAGCCCGG CTCTCTGTACGTGTCTTACGAATGGGTGACCACCGACAAGTT CAACGGCGACGACTCTGAGCACTGCGACGTGATCCAGGGCA TCGAGCGAGGCGACGCTCTGCCCGGCCTGCGACGATACGAC GAGATCTCTGAGGCCGCCAAGAAGGTGGGCTTCGAGATCGT GGACGAGCGAGATCTGGCCGCTCCTCCTGCTAAGCCCTGGT GGGACCGACTGAAGATGGGCCGAATCGCCTACTGGCGAAAC CACATCGTGGTGACCGTGCTGGCCGCCATCGGCGTGGCTCC CAAGGGCACCGTGGACGTGCACGACATGCTGTTCAAGACCG CCGACTACCTGACTCGAGGCGGCGAGTCTGGCATTTTCTCTC CCATGCACATGATCCTGTGTCGAAAGCCCGTGGACGCCAAGT CTGACTCTTAA

Table 4b: Codon-optimised sequences of heterologous genes

Gene	**Nucleic acid sequence, codon optimised for *Yarrowia lipolytica***
Danio rerio delta-24(25) sterol reductase	ATGGACCCTCTGCTGTACCTCGGCGGCCTGGCCGTGCTGTTCCTGATCTGGAT CAAGGTGAAGGGCCTCGAGTACGTGATCATCCACCAGAGATGGATCTTCGTGT GCCTGTTCCTGCTGCCTCTGTCTGTGGTGTTCGACGTGTACTACCACCTCCGAG CCTGGATCATCTTCAAGATGTGCTCTGCTCCCAAGCAGCACGACCAGCGAGTG CGAGACATCCAGCGACAGGTGCGAGAGTGGCGAAAGGACGGCGGCAAGAAGT ACATGTGCACCGGACGACCCGGCTGGCTGACCGTGTCTCTGCGAGTGGGCAA GTACAAGAAGACCCACAAGAACATCATGATCAACATGATGGACATCCTCGAGGT GGACACCAAGCGAAAGGTGGTGCGAGTCGAGCCCCTGGCCAACATGGGCCAA GTGACCGCTCTGCTGAACTCTATCGGCTGGACCCTGCCTGTGCTGCCCGAGCT GGACGACCTGACCGTCGGCGGACTGGTGATGGGAACCGGCATCGAGTCCTCT TCTCACATCTACGGCCTGTTCCAGCACATCTGCGTGGCCTTCGAGCTGGTGCT GGCCGACGGCTCTCTGGTCCGATGCACCGAGAAGGAAAACTCTGACCTGTTCT ACGCTGTGCCCTGGTCTTGCGGAACCCTGGGCTTCCTGGTCGCCGCCGAGATC CGAATCATCCCCGCTCAGAAGTGGGTCAAGCTGCACTACGAGCCCGTGCGAGG CCTGGACGCCATCTGCAAGAAGTTCGCCGAGGAATCTGCCAACAAGGAAAACC AGTTCGTCGAGGGCCTGCAGTACTCTCGAGATGAGGCCGTGATCATGACCGGC GTGATGACCGACCACGCTGAGCCCGACAAGACCAACTGCATCGGCTACTACTA CAAGCCCTGGTTCTTCCGACACGTCGAGTCTTTTCTGAAGCAGAACCGAGTGG CCGTCGAGTACATTCCCCTGCGACACTACTACCATCGACACACCCGATCTATTT TCTGGGAGCTGCAGGACATCATCCCCTTCGGCAACAACCCTCTGTTCCGATAC GTGTTCGGCTGGATGGTGCCTCCTAAGATCTCCCTGCTGAAGCTGACCCAGGG CGAGACTATCCGAAAGCTGTACGAGCAGCACCACGTCGTCCAGGACATGCTGG TGCCCATGAAGGACATCAAGGCCGCCATTCAGCGATTCCACGAGGACATCCAC GTGTACCCTCTGTGGCTGTGCCCCTTTCTGCTGCCCAACCAGCCTGGCATGGT GCACCCCAAGGGCGACGAGGACGAGCTGTACGTGGACATCGGCGCCTACGGC GAGCCCAAGGTCAAGCACTTCGAGGCCACCTCTTCTACCCGACAGCTCGAGAA GTTCGTGCGAGATGTGCACGGCTTCCAGATGCTGTACGCCGACGTCTACATGG

	AACGAAAGGAATTCTGGGAGATGTTCGACGGCACCCTGTACCACAAGCTGCGA GAGGAACTGGGCTGCAAGGACGCTTTCCCCGAGGTGTTTGACAAGATCTGCAA GTCTGCCCGACACTAA
Mus musculus delta-24 (25) sterol reductase	ATGGAACCCGCCGTGTCTCTGGCCGTGTGCGCCCTGCTGTTCCTGCTGTGGGT GCGAGTGAAGGGCCTCGAGTTCGTGCTGATCCACCAGAGATGGGTGTTCGTGT GCCTGTTTCTGCTGCCCCTGTCTCTGATCTTCGACATCTACTACTACGTGCGAG CCTGGGTCGTGTTCAAGCTGTCCTCTGCTCCCCGACTGCACGAGCAGCGAGTG CGAGACATCCAAAAGCAGGTCCGAGAGTGGAAGGAACAGGGCTCTAAGACCTT CATGTGCACCGGACGACCCGGCTGGCTGACCGTGTCGCTGCGAGTGGGCAAG TACAAGAAGACCCACAAGAACATCATGATCAACCTGATGGACATCCTCGAGGTG GACACCAAGAAGCAGATCGTCCGAGTCGAGCCCCTGGTGTCTATGGGCCAAGT GACCGCTCTGCTGAACTCTATCGGCTGGACCCTGCCTGTGCTGCCCGAGCTGG ACGACCTGACCGTCGGCGGCCTGATCATGGGAACCGGCATCGAGTCCTCTTCG CACAAGTACGGCCTGTTCCAGCACATCTGCACCGCCTACGAGCTGATCCTGGC CGACGGCTCTTTCGTCCGATGCACCCCTTCTGAGAACTCTGACCTGTTCTACGC TGTGCCCTGGTCTTGCGGAACCCTGGGCTTCCTGGTGGCCGCCGAGATCCGAA TCATCCCCGCCAAGAAGTACGTCAAGCTGCGATTCGAGCCCGTGCGAGGACTG GAAGCCATCTGCGAGAAGTTCACCCGAGAGTCTCAGCGACTCGAGAACCACTT CGTCGAGGGCCTGCTGTACTCTCTGGACGAGGCCGTGATCATGACCGGCGTG ATGACCGACGACGTCGAGCCCTCTAAGCTGAACTCCATTGGCTCTTACTACAAG CCCTGGTTCTTCAAGCACGTCGAGAACTACCTCAAGACCAACCGAGAGGGACT CGAGTACATTCCCCTGCGACACTACTACCACCGACACACCCGATCTATTTTCTG GGAGCTGCAGGACATCATCCCCTTCGGCAACAACCCCATCTTCCGATACCTGTT CGGCTGGATGGTGCCTCCTAAGATCTCCCTGCTGAAGCTGACCCAGGGCGAGA CTCTGCGAAAGCTGTACGAGCAGCACCACGTCGTCCAGGACATGCTGGTGCCC ATGAAGTGCATGTCTCAGGCCCTGCACACCTTCCAGAACGACATCCACGTGTAC CCCATCTGGCTGTGCCCCTTCATTCTGCCCTCTCAGCCCGGCCTGGTGCACCC CAAGGGCGACGAGGCTGAGCTGTACGTGGACATCGGCGCCTACGGCGAGCCC CGAGTGAAGCACTTCGAGGCCCGATCTTGCATGCGACAGCTCGAGAAGTTTGT GCGATCTGTGCACGGCTTCCAGATGCTGTACGCCGACTGCTACATGAACCGAG AAGAGTTCTGGGAGATGTTCGACGGATCTCTGTACCACAAGCTCCGAAAGCAG CTGGGCTGCCAGGACGCTTTCCCCGAGGTGTACGACAAGATCTGCAAGGCCG CTCGACACTAA
Solanum tuberosum delta-24(28) sterol reductase	ATGACCGACGTCCAGGCCCCTCCTCGACCCAAGCGAAAGAAGAACATCATGGA CCTCCTCGTCCAGTTCCGATGGATCGTCGTCATCTTCGTCGTCCTCCCCCTCTC CTTCCTCTACTACTTCTCCATCTACGTCGGTGACGTCCGATCCGAGTGCAAGTC CTACAAGCAGCGACAGAAGGAGCACGACGAGAACGTCAAGAAGGTCGTCAAGC GACTCAAGGACCGAAACGCCTCCAAGGACGGTCTGGTCTGCACCGCCCGAAA GCCCTGGGTCGCTGTCGGTATGCGAAACGTCGACTACAAGCGAGCCCGACACT TCGAGGTCGACCTCTCCCCCTTCCGAAACGTCCTCAACATCGACACCGAGCGA ATGATCGCCAAGGTCGAGCCCCTCGTCAACATGGGTCAGATCTCCCGAGTCAC CGTCCCCATGAACGTCTCCCTCGCCGTCGTCGCTGAGCTCGACGACCTCACCG TCGGTGGTCTGATCAACGGTTACGGTATCGAGGGTTCCTCCCACATCTACGGT CTGTTCTCCGACACCGTCGTCTCCTACGAGGTCGTCCTCGCCGACGGTCAGGT CGTCCGAGCCACCAAGGACAACGAGTACTCCGACCTCTTCTACGCCATCCCCT GGTCCCAGGGTACCCTCGGTCTGCTCGTCTCCGCCGAGATCAAGCTCATCCCC ATCAAGGAGTACATGAAGCTCACCTACAAGCCCGTCGTCGGTAACCTCAAGGA GATCGCCCAGGCCTACATCGACTCCTTCTCCCCCAAGGACGGTGACCAGGACA ACCGAGAGAAGGTCCCCGACTTCGTCGAGACTATGGTCTACACCCCCACCGAG GCTGTCTGCATGACCGGTCGATACGCCTCCAAGGAGGAGGCCAAGAAGAAGG GTAACGTCATCAACAACGTCGGTTGGTGGTTCAAGACCTGGTTCTACCAGCACG CCCAGACCGCTCTCAAGAAGGGTGAGTTCGTCGAGTACATCCCCACCCGAGAG TACTACCACCGACACACCCGATGCCTCTACTGGGAGGGTAAGCTCATCCTCCC CTTCGGTGACCAGTGGTGGTTCCGATTCTTCTTCGGTTGGGCCATGCCCCCTAA GGTTTCCCTCCTCAAGGCCACCCAGGGTGAGTACATCCGAAACTACTACCACG AGAACCACGTCATCCAGGACATGCTCGTCCCCCTCTACAAGGTCGGTGACGCC CTCGAGTGGGTCAACCGAGAGATGGAGGTCTACCCCCTCTGGCTCTGCCCCCA CCGACTCTACCGACTCCCCCTCAAGACCATGGTCTACCCCGAGCCTGGTTTCG AGCTCCACAAGCGACAGGGTGACACCAAGTACGCCCAGATGTACACCGACGTC GGTGTCTACTACGCCCCCGGTCCCATCCTCCGAGGTGAGGTCTTCGACGGTAT CGAGGCCGTCCGAAAGCTCGAGTCCTGGCTCATCGAGAACCACGGTTTCCAGC

	CCCAGTACGCCGTCTCCGAGCTCACCGAGAAGAACTTCTGGCGAATGTTCGAC GGTTCCCTCTACGAGAACTGCCGAAAGAAGTACCGAGCCATCGGTACCTTCAT GTCCGTCTACTACAAGTCCAAGAAGGGTAAGAAGACCGAGAAGGAGGTCCAGG ACGCCGAGCAGGAGACTGCCGAGGTCGAGACTCCCGAGGTCGACGAGCCCGA GGACTAA
Arabidopsis thaliana C-28 sterol methyl-transferase	ATGGACTCTCTGACCCTGTTCTTCACCGGCGCTCTGGTGGCCGTGGGAATCTA CTGGTTCCTGTGCGTGCTGGGACCCGCCGAGCGAAAGGGCAAGCGAGCCGTG GACCTGTCTGGCGGCTCTATCTCTGCCGAGAAGGTGCAGGACAACTACAAGCA GTACTGGTCGTTCTTCCGACGACCTAAGGAAATCGAGACTGCTGAGAAGGTCC CCGACTTCGTGGACACCTTCTACAACCTGGTGACCGACATCTACGAGTGGGGC TGGGGCCAGTCTTTCCACTTCTCTCCCTCGATTCCCGGCAAGTCTCACAAGGAC GCTACCCGACTGCACGAAGAGATGGCCGTCGACCTGATCCAGGTGAAGCCCG GCCAAAAGATCCTGGACGTCGGCTGCGGCGTCGGCGGACCCATGCGAGCCAT TGCCTCTCACTCTCGAGCCAACGTGGTGGGCATCACCATCAACGAGTACCAGG TGAACCGAGCCAGACTGCACAACAAGAAGGCCGGACTGGACGCCCTGTGCGA GGTGGTGTGCGGCAACTTCCTGCAGATGCCCTTCGACGACAACTCTTTCGACG GCGCCTACTCTATCGAGGCCACCTGTCACGCTCCCAAGCTGGAAGAGGTGTAC GCCGAGATCTACCGAGTGCTGAAGCCTGGCTCTATGTACGTGTCTTACGAATG GGTGACCACCGAGAAGTTCAAGGCCGAGGACGACGAGCACGTCGAGGTGATC CAGGGCATCGAGCGAGGCGACGCTCTGCCCGGCCTGCGAGCCTACGTGGACA TTGCCGAGACAGCCAAGAAGGTGGGCTTCGAGATCGTGAAGGAAAAGGACCTG GCCTCGCCTCCTGCTGAGCCCTGGTGGACCCGACTGAAGATGGGCCGACTGG CCTACTGGCGAAACCACATCGTGGTGCAGATCCTGTCTGCCGTGGGCGTCGCC CCTAAGGGCACCGTGGACGTGCACGAGATGCTGTTCAAGACCGCCGACTACCT GACTCGAGGCGGCGAGACTGGCATTTTCTCTCCCATGCACATGATCCTGTGTC GAAAGCCCGAGTCTCCCGAGGAATCTTCTTAA
Amborella trichopoda C-28 sterol methyl-transferase	ATGGAGACTCTGGCCGCCGTTGTCACCCTGGGTCTGCTCGCTGCCGGCCTCTA CTGGTTCGTCTGCCTGCTGGGTTCTGCCGAGCAGAAAGGTAAGCACGCCTCTG AGCTGTCTGGAGGCTCTCTGGGCCGAGAGCAGGTCGCCTCCACCTACCGACA GTACTGGACCTTCTTCCGAAAGCCCAAGGAAATTGAACACGCCGACCGAGTGC CCGACCTCGTCGACTCGTTTTACAACCTGGTGACCGACATCTACGAGTGGGGC TGGGGACAGTCTTTCCACTTCTCTCCTTCTCTGCCCGGACATTCCCATGCCGCC GCTACCCGAGCTCACGAGGAGATGGCCGCTAACCTTCTGAAGCTTGGCCCCGC CATGAAGGTCCTTGACGCTGGATGCGGCGTGGGTGGCCCCATGCGAACTATCG CTACTCACTCCGGCGCTAACGTCGTCGGCATCACCATCAACGAGTACCAGGTC GGCCGAGCCCGACTGCACAACATCAAGGCCGGTCTGGACAAGCTGTGCGAAG TCGTTTGCGGTGATTTCCTGCACATGCCCTTCGATGCTGAGTCCTTCGACGCCG CCTACTCCATCGAGGCCACCTGCCACGCTCCCAAGCTCTCCGAGGTCTACGCC GAGATTTTCCGGGTTCTGAAGCCCGGTGCCCTCTACGTGACCTACGAATGGGT CACCACCCCCAAGTTTCAGCCCGACAATTCCGAACATCTGGAGATCGTCCAGG GCATCGAGCGAGGTAACGCCCTTCCCGGTCTCCGACGACAGGACGAGGTCGC CGAAATCGCCAAGGGCGTCGGCTTCGAGCTGGTTGAAGAGCGGGACCTGGCC CTACCCCCTGCCCTGCCTTGGTGGACTCGACTTAAGATGGGTCGAGTCGCCTA CTTTCGAAACCACGTGGTCGTCTGGGTCCTGACCATGGTCCGAATTGCCCCTAA GGGCGTGGACGACGTGCACGAGATGCTGTTTCATACCGCCCACCACCTGACTC GAGGCGGTCAGACCGGCATCTTCACCCCCATGCACATGATTCTGCTTCGAAAA CCTAACAACGCTGCTCCCGCCTGCTAA

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Table 5a: Yeast strains

			Elements used to construct strain	
Strain name	**Genotype**	**Parent strain /Reference**	**gRNA vector**	**Integration vector/BioBrick**
ST4840		*Y. lipolytica* Y-17536 (ATCC© 34088 ™) from the ARS culture collection		
ST4842	MATa	*Y. lipolytica* W29 (MatA, ATCC©20460™) strain Y-63746 from the ARS culture collection		
ST6512	MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2	ST4842/ (Marella *et al*, 2020)		
ST8980	ST8980 MATa ku70Δ::PrTEF1->Cas9-TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt- >ERG12	ST6512/ (Arnesen *et al*, 2020); available from Euroscarf (accession no. Y41408)	pCfB6627	pCfB8822
ST9027	MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 IntC_3-SeACS<-PrGPDPrTefInt->YlACL1	ST8980/ (Arnesen *et al*, 2020)	pCfB8856	pCfB8823
ST9100	MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 IntC_3-SeACS<-PrGPDPrTefInt->YlACL1 IntD_1-IDI1<- PrGPD-PrTefInt->ERG20	ST9027/ (Arnesen *et al*, 2020)		
ST3683	mus51Δ, nugm-Htg2, ndh2i, lys11-, leu2-, ura3-, MatB	(Arnesen *et al*, 2020; Angerer *et al*, 2014) *Y. lipolytica* GB20 was a kind gift from Volker Zickermann.		
ST10924	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_4-PrTefInt->DHCR7_Esiliculosus	ST9100	pCfB8861 (pHyg-YLgRNA2_IntE_4)	pCfB10252 (IntE4_PrTEF->DHCR7_Esilicul)

ST10934	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_4-PrTefInt->DHCR7_Esiliculosus ERG5_tPr48NatMX	ST10924		pCfB10367 (Erg5_tPr48)
ST11005	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC	ST9100		pCfB10494 (IntE3_PrGPAT->TtSTC)
ST11014	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5_HphMX	ST11005		BB5098 (Erg5_HphMX_KO)
ST11027	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5	ST11014		pCfB6611 (pNat-PrExp-Cre)
ST11028	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 Δerg5_HphMX Δerg4_NatMX	ST11014		BB5246 (Erg4_NatMX_KO)
ST11325	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt-	ST11027		BB5099 (Erg6_HphMX_KO)

	>ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg6_HphMX			
ST11326	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 -erg4_NatMX	ST6512		BB5246 (Erg4_NatMX_KO)
ST11327	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 -erg5_NatMX	ST6512		BB5247 (Erg5_NatMX_KO)
ST11040	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX	ST11027		BB5097 (Erg4_HphMX_KO)
ST11066	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Stuberosum	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10249 (IntE4_PrTEF->DHCR7_Stuberosum)
ST11067	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Drerio	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10250 (IntE4_PrTEF->DHCR7_Drerio)
ST11068	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10251 (IntE4_PrTEF->DHCR7_Ldrancou)

	IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Ldrancou			
ST11069	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Esilicul	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10252 (IntE4_PrTEF->DHCR7_Esilicul)
ST11070	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Cprotoch	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10253 (IntE4_PrTEF->DHCR7_Cprotoch)
ST11071	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Csubellip	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10254 (IntE4_PrTEF->DHCR7_Csubellip)
ST11072	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Mverticillata	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10255 (IntE4_PrTEF->DHCR7_Mverticillata)
ST11073	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt-	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10256 (IntE4_PrTEF->DHCR7_Gsoja)

	>ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Gsoja			
ST11074	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Tsp	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)
ST11075	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 IntE_4-PrTEF->DHCR7_Wchondrophi	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10258 (IntE4_PrTEF->DHCR7_Wchondrophi)
ST11056	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Stuberosum	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10249 (IntE4_PrTEF->DHCR7_Stuberosum)
ST11057	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Drerio	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10250 (IntE4_PrTEF->DHCR7_Drerio)

ST11058	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Ldrancou	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10251 (IntE4_PrTEF->DHCR7_Ldrancou)
ST11059	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Esilicul	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10252 (IntE4_PrTEF->DHCR7_Esilicul)
ST11060	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Cprotoch	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10253 (IntE4_PrTEF->DHCR7_Cprotoch)
ST11061	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Csubellip	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10254 (IntE4_PrTEF->DHCR7_Csubellip)
ST11062	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10255 (IntE4_PrTEF->DHCR7_Mvertic illata)

	IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Mverticillata			
ST11063	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Gsoja	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10256 (IntE4_PrTEF->DHCR7_Gsoja)
ST11064	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Tsp	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)
ST11065	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Δerg5 Δerg4_HphMX IntE_4-PrTEF->DHCR7_Wchondrophi	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10258 (IntE4_PrTEF->DHCR7_Wchondrophi)
ST10924	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_4-PrTefInt->DHCR7_Esiliculosus	ST9100	pCfB8861 (pHyg-YLgRNA2_IntE_4)	pCfB10252 (IntE4_PrTEF->DHCR7_Esiliculosus)

ST10934	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_4-PrTefInt->DHCR7_Esiliculosus HygMX-ERG5_tPr48	ST10924		pCfB10367 (Erg5_trPr48)
ST11196	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_4-PrTefInt->DHCR7_Esiliculosus ΔPAH1	ST10924		pCfB7254 (gRNA for PAH1 deletion Y.L); BB5314 (PAH1 repair template)
ST11197	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC IntE_4-PrTEF->DHCR7_Esilicul ΔERG5 ΔPAH1	ST11069		pCfB7254 (gRNA for PAH1 deletion Y.L); BB5314 (PAH1 repair template)
ST11337	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntF3_PrDGA1->ERG4	ST11064	pBP8003 (pNat-YLgRNA4-IntF_3)	pCfB10841 (IntF3_PrDGA1->ERG4)
ST11338	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-	ST11064	pBP8003 (pNat-YLgRNA4-IntF_3)	pCfB10842 (IntF3_PrGPAT->ERG4)

	PrTEF->DHCR7_Tsp IntF3_PrGPAT->ERG4			
ST11378	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrDGA1->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic	ST11337	pCfB10783 (pNatYlgRNA-IntE_5)	pCfB10853 (IntE5_PrGPAT->DHCR24_Slycopersic)
ST11340	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrGPAT->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic	ST11338	pCfB10783 (pNatYlgRNA-IntE_5)	pCfB10853 (IntE5_PrGPAT->DHCR24_Slycopersic)
ST11362	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrGPAT->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic IntE1_PrGPAT->SMT2_Cquinoa	ST11340	pCfB6633 (pNat-YLgRNA2_IntE_1)	pCfB10851 (IntE1_PrGPAT->SMT2_Cquinoa)
ST11441	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-NatMX -erg5 -erg4_HphMX	ST11362		pCfB4783 (Int_3 NatMX)

	IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrGPAT->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic IntE1_PrGPAT->SMT2_Cquinoa			
ST11540	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4 IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrDGA1->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic	ST11378		pCfB6611 (pNat-PrExp-Cre
ST11541	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-NatMX-PrGPAT->SMT2_Cquinoa -erg5 -erg4 IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrDGA1->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic	ST11540		pCfB10919 (Int_3 NatMX_PrGPAT->SMT2_Cquinoa)
ST11542	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->SMT2_Cquinoa -erg5 -erg4 IntE_4-PrTEF->DHCR7_Tsp IntF_3-PrDGA1->ERG4 IntE_5-PrGPAT->DHCR24_Slycopersic	ST11541		pCfB6612 (pHph-PrExp-Cre)

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Table 5b: Yeast strains

			Elements used to construct strain	
Strain name	**Genotype**	**Parent strain/ Reference**	**gRNA vector**	**Integration vector/BioBrick**
ST4842	MATa	*Y. lipolytica* W29 (MatA, ATCC©20460™) strain Y-63746 from the ARS culture collection		
ST6512	MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2	ST4842/ (Marella *et al*, 2020)		
ST9100	MATa ku70Δ::PrTEF1->Cas9- TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 IntC_3-SeACS<-PrGPDPrTefInt->YlACL1 IntD_1-IDI1<- PrGPD-PrTefInt->ERG20	ST6512/ (Arnesen *et al*, 2020)		
ST11005	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC	ST9100	pCfB6637 (pNat-YLgRNA3_IntE_3)	pCfB10494 (IntE3_PrGPAT->TtSTC)
ST11014	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Derg5_HphMX	ST11005		BB5098 (Erg5_HphMX_KO)
ST11027	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Derg5	ST11014		pCfB6611 (pNat-PrExp-Cre)
ST11040	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT-	ST11027		BB5097 (Erg4_HphMX_KO)

	>TtSTC Derg5 Derg4_HphMX			
ST11325	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg6_HphMX	ST11027		BB5099 (Erg6_HphMX_KO)
ST11330	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg6	ST11325		pCfB6611 (pNat-PrExp-Cre)
ST11071	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Derg5 IntE_4-PrTEF->DHCR7_Csubellip	ST11027	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10254 (IntE4_PrTEF->DHCR7_Csubellip)
ST11064	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Derg5 Derg4_HphMX IntE_4-PrTEF->DHCR7_Tsp	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)
ST11804	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 IntE_4-PrTEF->DHCR7_Csubellip IntE_1-PrTEF->SMT2_Cquinoa	ST11071	pCfB6633 (pNat-YLgRNA2_IntE_1)	pCfB10311 (intE1_PrTEF->SMT2_Cquinoa)

ST12139	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 IntE_4-PrTEF->DHCR7_Csubellip IntE_1-PrTEF->SMT2_Cquinoa IntF3_SMT_Ath<-PrGPD_TEFi->SMT_Atr	ST11804	pBP8003 (pNat-YLgRNA4-IntF_3)	pCfB11486 (IntF3_SMT_Ath<-PrGPD_TEFi->SMT_Atr)
ST11803	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntE_1-PrTEF->SMT2_Cquinoa	ST11064	pCfB6633 (pNat-YLgRNA2_IntE_1)	pCfB10311 (intE1_PrTEF->SMT2_Cquinoa)
ST12108	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntE_1-PrTEF->SMT2_Cquinoa IntF3_SMT_Ath<-PrGPD_TEFi->SMT_Atr	ST11803	pBP8003 (pNat-YLgRNA4-IntF_3)	pCfB11486 (IntF3_SMT_Ath<-PrGPD_TEFi->SMT_Atr)
ST11346	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg6 IntE_4-PrTEF->DHCR7_Ldrancou	ST11330	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10251 (intE4_PrTEF->DHCR7_Ldrancou)
ST11829	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-	ST11346	pCfB6633 (pNat-	pCfB10293 (intE1_PrTEF->DHCR24_Drerio)

	HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg6 IntE_4-PrTEF->DHCR7_Ldrancou IntE1_PrTEF->DHCR24_Drerio		YLgRNA2_IntE_1)	
ST11830	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg6 IntE_4-PrTEF->DHCR7_Ldrancou IntE1_PrTEF->DHCR24_Mmusculus	ST11346	pCfB6633 (pNat-YLgRNA2_IntE_1)	pCfB10298 (intE1_PrTEF->DHCR24_Mmusculus)

Table 5c: Yeast strains

			Elements used to construct strain	
Strain name	**Genotype**	**Parent strain/ Reference**	**gRNA vector**	**Integration vector/BioBrick**
ST4842	MATa	*Y. lipolytica* W29 (MatA, ATCC©20460™) strain Y-63746 from the ARS culture collection		
ST6512	MATa ku70Δ::PrTEF1->Cas9-TTef12::PrGPD->DsdA-TLip2	ST4842/ (Marella *et al*, 2020)		
ST9100	MATa ku70Δ::PrTEF1->Cas9-TTef12::PrGPD->DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt- >ERG12 IntC_3-SeACS<-PrGPDPrTefInt->YlACL1 IntD_1-IDI1<- PrGPD-PrTefInt->ERG20	ST6512/ (Arnesen *et al*, 2020)		
ST11005	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-	ST9100	pCfB6637 (pNat-YLgRNA3_IntE_3)	pCfB10494 (IntE3_PrGPAT->TtSTC)

	HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC			
ST11014	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Derg5_HphMX	ST11005		BB5098 (Erg5_HphMX_KO)
ST11027	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC Derg5	ST11014		pCfB6611 (pNat-PrExp-Cre)
ST11040	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX	ST11027		BB5097 (Erg4_HphMX_KO)
ST11064	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-	ST11040	pCfB6638 (pNat-YLgRNA2_IntE_4)	pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)

	SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp			
ST11943	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntF3_PrDGA1->StSSR1	ST11064	pBP8003 (pNat-YLgRNA4-IntF_3)	pCfB10948 (IntF3_PrDGA1->StSSR1)
ST12140	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-PrTEF->DHCR7_Tsp IntF3_PrDGA1->StSSR1 IntE1_PrGPAT->SMT2_Cq	ST11943	pCfB6633 (pNat-YLgRNA2_IntE_1)	pCfB10851 (IntE1_PrGPAT->SMT2_Cquinoa)
ST12178	MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20 IntE_3-PrGPAT->TtSTC -erg5 -erg4_HphMX IntE_4-	ST12140	pCfB10783 (pNatYlgRNA-IntE_5)	pCfB10853 (IntE5_PrGPAT->DHCR24_Slycopersic)

	PrTEF->DHCR7_Tsp IntF3_PrDGA1->StSSR1 IntE1_PrGPAT->SMT2_Cq IntE5_PrGPAT->DHCR24_Sl			

Foundational genome engineering toolbox for *Y. lipolytica*

The engineering undertaken for *Y. lipolytica* strain production as described herein below utilised the EasyClone YALI toolbox with USER® cloning for Y. lipolytica as established by Holkenbrink et al. and described in Holkenbrink et al. EasyCloneYALI:CRISPR/Cas9-Based Synthetic Toolbox for Engineering of the Yeast *Yarrowia lipolytica,* Biotechnol. J. (2018). This approach relies on a toolbox of genome engineering components all of which are attainable with reference to known construct or sequence information including a set of integrative expression vectors which allow expression of one or two genes per vector and integration into highly expressed intergenic genome sites (see for example Addgene for vector information).The exact genome locations of these integration sites are listed in Table S5 of the supporting information of the Holkenbrink et al. 2018 paper and are also listed in Table 18 below. For the purpose of the *Y. lipolytica* engineering disclosed herein, reference to integrative expression vectors and other information available from the Holkenbrink et al. 2018 paper is supplemented with information on plasmids required and their construction with reference to biobricks (DNA fragments for cloning) and their derivation by PCR amplification. Heterologous genes for expression in *Y. lipolytica* were codon-optimised for this yeast and obtained as synthetic DNA fragments (see Table 4 above).

Example 1 - Construction of *Yarrowia lipolytica* platform strain for sterol production

Materials and Methods

Strains, culture conditions and chemicals

Escherichia coli strain DH5α was used for plasmid construction. *E. coli* was grown at 37 °C and 300 rpm in Lysogeny Broth (LB) liquid medium and at 37 °C on LB solid medium plates supplemented with 20 g/l agar. Ampicillin was supplemented at a concentration of 100 mg/l for plasmid selection.

The *Yarrowia lipolytica* W29 derived strain ST6512 (Mata ku70Δ:PrTEF1->Cas9-TTef12:PrGPD->DsdA-TLip2) was obtained from Irina Borodina (Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Copenhagen).The strain may also be requested from Euroscarf (accession no. Y41408). This strain is engineered from the commercially available *Y. lipolytica* W29 strain Y-63746 (MatA, ATCC® 20460™/ ARS Culture Collection, NCAUR, United States) as described fully in Marella *et al.* (2020) Metabol. Eng. The strain ST6512 was used to construct the platform strain (designated ST9100). All strains are detailed in Table 5.

Y. lipolytica was grown at 30°C on yeast extract peptone dextrose (YPD) media containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose, supplemented with 20 g/l agar for preparation of

solid media. For selection, either nourseothricin (250 mg/l) or hygromycin (400 mg/l) was added to the media.

Cultivation of strains for sterol production was performed in yeast extract peptone medium containing 80 g/l glucose.

Chemicals were obtained, if not indicated otherwise, from Sigma-Aldrich. Nourseothricin was purchased from Jena BioScience GmbH (Germany).

Plasmid construction

Native gene sequences were amplified from CLIB122 derived strain ST3683 gDNA. Strain ST9204 as described in Arnesen *et al*. 2020, is derived from ST9100. Hence, ST9204 includes all the genomic features of ST9100. Sequences required to construct ST9100 can therefore be equivalently amplified from ST9204 gDNA. The strain ST9204 may be requested from Euroscarf (accession no. Y41404).

The gene encoding *Salmonella enterica* acetyl-CoA synthetase (*SeACS*, GenBank accession: WP_000083882.1) was codon-optimized for *Y. lipolytica* and synthesised as GeneArt String DNA fragments by ThermoFisher Scientific (Table 4).

The plasmids, biobricks, and primers used in this study are listed in Tables 6-8, respectively. As indicated above, the biobricks were amplified by PCR. The PCR mix contained 32 μl water, 10 μl high fidelity Phusion® polymerase buffer (5x), 1 μl 10mM dNTP, 1.5 μl $MgCl_2$, 2.5 μl 10 μM Fw primer, 2.5 μl 10 μM Rv primer, 0.2 μl Phusion® U polymerase, and 10 ng DNA template. Reactions were multiplexed 8x per BioBrick. The cycling program was: 98°C for 1 min, 30 cycles of [98°C for 10 sec, gradient from 50 to 58°C for 20 sec, 72°C for 30 s/kb], 72°C for 5 min, pause at 10°C. The gene fragments were purified from agarose gels using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Biobricks were assembled into the EasyCloneYALI vectors with USER cloning (Holkenbrink *et al*, 2018). The USER reactions were transformed into *E. coli* and correct assemblies were verified by Sanger sequencing (Eurofins).

Table 6: Plasmids for construction of *Yarrowia lipolytica* platform strain for sterol production

Plasmid name	Parent plasmid/ Reference	BioBricks
pCfB8843 (pORI1001-Hyg-CEN1-USER)	(Arnesen *et al*, 2020)	BB3924, BB3925
pCfB6682 (pIntC_2-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6371 (pIntC_3-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6684 (pIntD_1-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6627 (pNat-YLgRNA2_IntC_2)	(Holkenbrink *et al*, 2018)	
pCfB6631 (pNat-YLgRNA2_IntD_1)	(Holkenbrink *et al*, 2018)	
pCfB8856 (pHphMX-YLgRNA2_IntC_3)	pCfB8843/ (Arnesen *et al*, 2020)	BB3927
pCfB8822 (IntC_2-HMG1<-PrGPD-PrTefInt->ERG12)	pCfB6682/ (Arnesen *et al*, 2020)	BB3865, BB3866, BB3867
pCfB8823 (IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1)	pCfB6371/ (Arnesen *et al*, 2020)	BB3865, BB3868, BB3869
pCfB8878 (IntD_1-IDI1<-PrGPD-PrTefInt- >ERG20)	pCfB6684/ (Arnesen *et al*, 2020)	BB3865, BB3870, BB3871
pCfB6605 (pIntE-4-Hph-PrExp->YlSQS1)	(Arnesen *et al*, 2020)	

pCfB6630 (pNat-YLgRNA3_IntC_3)	(Holkenbrink *et al*, 2018)	
pCfB7063 (prDNA-Ura3d1-TPex20+PTEfintron+CrtW+TLip2)	(Kildegaard *et al*, 2017)	
pCfB5119 (pIntB-HphMx-YlHMG1<-PrGPDPrFBA1->YlGGS1)	(Kildegaard *et al*, 2017)	
pCfB6620 (pORI1001-Nat-CEN1-USER-IDI1<-PrEXP-PrGPD->ERG20)	(Arnesen *et al*, 2020)	

Table 7: Biobricks for construction of *Yarrowia lipolytica* platform strain for sterol production

BioBrick name	Template/Reference	Forward primer	Reverse primer
BB3924 (Episomal vector backbone w/o HphMX)	pCFB3405	PR-23934	PR-10593
BB3925 (HphMX-TTef1 insert)	pCfB6605	PR-23935	PR-23936
BB3927 (gRNA-cassette IntC_3)	pCfB6630	PR-10607	PR-10604
BB3863 (Tefint(PrGDPfusion)->)	pCfB7063	PR-24013	PR-18214
BB3864 (<-PrGDP)	pCfB5119	PR-15528	PR-15529
BB3865 (<-PrGDP_Tefint->)	BB3863, BB3864	PR-15528	PR-18214
BB3866 (<-HMG1)	pCfB5119	PR-23753	PR-23752
BB3867 (ERG12->)	ST3683 gDNA	PR-24014	PR-24015
BB3868 (<-YlOpSeACS)	*Salmonella enterica* acetyl-CoA synthetase *Y. lipolytica* codon optimised	PR-24016	PR-24017
BB3869 (YlACL1->)	ST3683 gDNA	PR-24018	PR-24019
BB3870 (<-IDI1)	ST3683 gDNA	PR-24020	PR-24021
BB3871 (ERG20->)	pCfB6620	PR-24022	PR-24023

Table 8: Primers for construction of *Yarrowia lipolytica* platform strain for sterol production

Primer name	Sequence (5' → 3')
PR-23934 (PrEXP_for-HpHMX_rv)	ACCCATTGCTGUAGATATGTCTTGTGTGTAAGGGGG
PR-10593 (Fragment2EpiVecYL _fw)	AGCAGGCTUGGAGGCGACGTGGCAG
PR-23935 (HphMX_for-PrEXP1_fw)	ACAGCAATGGGUAAAAAGCCTGAACTCACCGC
PR-23936 (TTef1_rv)	AAGCCTGCUGAATTCGGACACGGGCAT
PR-10604 (tracrRNA _rev)	CACGCGAUACCGTACCCACACAAAAAAAGCACCACCGACTC
PR-10607 (PrtRNAGly _fw)	CGTGCGAUAGTGAATCATTGCTAACAGATC
PR-24013 (Tefint_Fuse_fw)	ATCAGTAGCUAGAGACCGGGTTGGCGGCG
PR-18214 (PTEFintron_USER_rv)	AGTACTGCAAAAAGUGCTG
PR-15528 (PrYlTDH1 _rev)	ATGACAGAUTGTTGATGTGTGTTTAATTCAAGAATG
PR-15529 (PrYlTDH1for fusion_fw)	AGCTACTGAUGACGCAGTAGGATGTCCTGCACGG
PR-23752 (YlHMG1 ->_U2_fw)	ATCTGTCAUGCCACAATGCTACAAGCAGCTATTGG
PR-23753 (YlHMG1 ->_U2_rv)	CACGCGAUCTATGACCGTATGCAAATATTCG

PR-24014 (ERG12_fw)	ACTTTTTGCAGTACUAACCGCAGGACTACATCATTTCGGCGCC
PR-24015 (ERG12_rev)	CACGCGAUCTAATGGGTCCAGGGACCG
PR-24016 (OpSeACS_fw)	CGTGCGAUTTAAGAGGGCATAGCAATGGCC
PR-24017 (OpSeACS_rev)	ATCTGTCAUGCCACAATGTCTCAGACCCACAAGCACG
PR-24018 (YlACL1_fw)	ACTTTTTGCAGTACUAACCGCAGTCAGCGAAATCCATTCACGAG
PR-24019 (YlACL1_rev)	CACGCGAUTTAAACTCCGAGAGGAGTGGAA
PR-24020 (YlIDI1_fw)	CGTGCGAUCTACTTGATCCACCGCCGAA
PR-24021 (YlIDI1_rev)	ATCTGTCAUGCCACAATGACGACGTCTTACAGCGA
PR-24022 (ERG20_fw)	ACTTTTTGCAGTACUAACCGCAGTCCAAGGCGAAATTCGAAAGC
PR-24023 (ERG20_rv)	CACGCGAUCTACTTCTGTCGCTTGTAAATCTT

Yeast transformation

The yeast vectors were integrated into different previously characterized intergenic loci in the *Y. lipolytica* genome as described in Holkenbrink *et al*, 2018; see also Table 18 below. Integration vectors were digested with *Not*I enzyme (New England BioLabs) at 37°C for 1 hr and the digested product purified from solution using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The purified DNA was transformed using the lithium acetate transformation protocol as described by Holkenbrink *et al*, 2018. Correct integration was verified by colony PCR using Taq DNA Polymerase Master Mix RED (Ampliqon) with vector-specific primers and primers complementary to the genomic region adjacent to the integration site as described by Holkenbrink et al.(2018).

Results

Yarrowia lipolytica strain ST9100 (MATa ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20) was constructed from ST6512 by consecutive rounds of engineering via firstly construction of ST8980 and then ST9027 as indicated in Table 5a above and also described in Arnesen et al. (2020) Front. Bioeng. Biotech. This was chosen as the platform *Y. lipolytica* strain for further sterol production studies for reasons given below.

Y. lipolytica is an oleaginous species with a high acetyl-coenzyme A (CoA) flux, the main precursor to the melavonate (MVA) and subsequent sterol pathways, which makes it valuable for sterol production. Two key approaches were used to develop the platform strain: improvement of the acetyl-CoA pool and up-regulation of the MVA-pathway to improve the accumulation of isopentyl diphosphate (IPP)/ dimethylallyl diphosphate (DMAPP). The acetyl-CoA pool was increased by overexpressing the native ATP citrate lyase 1 (*ACL*, YALI0_D24431g, *Y. lipolytica* CLIB122 genome assembly Dujon *et al*, 2004) and the *Salmonella enterica* acetyl-CoA synthetase (*SeACS*). In *Y. lipolytica*, Aclp generates acetyl-CoA and oxaloacetate from citrate, whilst *Se*Acsp produces acetyl-CoA from acetate and CoA. Flux through the melavonate pathway was increased by overexpression of native 3-hydroxy-3-methylglutaryl-CoA reductase (*HMG*, YALI0_E04807g), mevalonate kinase (*ERG12*, YALI0_B16038g), isopentyl diphosphate isomerase (*IDI*, YALI0_F04015g) and farnesyl diphosphate synthase (*ERG20*, YALI0_E05753g) genes.

References:

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Marella Er, Dahlin J, Dam MI, ter Horst J, Christensen HB, Sudarsan S, Wang G, Holkenbrink c & Borodina I (2020) A single-host fermentation process for the production of flavour lactones from non-hydroxylated fatty acids. Metabol. Eng. 61, 427-436

Example 2 - Engineering *Yarrowia lipolytica* strains incapable of synthesising ergosterol

Materials and Methods

Strains, culture conditions and chemicals

Escherichia coli strain DH5α was used for plasmid construction as noted in Example 1 above.

The *Y. lipolytica* strain ST4840 (ATCC© 34088 ™/Y-17536 ARS Culture Collection, NCAUR, United States), and W29-derived strains ST4842 (MatA, ATCC® 20460™/ Y-63746), ST6512 (MatA ku70Δ:PrTEF1->Cas9-TTef12:PrGPD->DsdA-TLip2, Marella et al. 2020) and ST9100 (MatA ku70Δ::PrTEF1-Cas9-TTef12::PrGPD-DsdA-TLip2 IntC_2-HMG1<-PrGPD-PrTefInt->ERG12 pCfB8823 IntC_3-SeACS<-PrGPD-PrTefInt->YlACL1 IntD_1-IDI1<-PrGPD-PrTefInt->ERG20, Arnesen *et al*, 2020) were obtained from Irina Borodina (Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Copenhagen). All strains are detailed in Table 5.

Y. lipolytica was grown at 30°C on yeast extract peptone dextrose (YPD) media containing 10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose, supplemented with 20 g/l agar for preparation of solid media. For selection, either nourseothricin (250 mg/l) or hygromycin (400 mg/l) was added to the media. Where indicated, 25 µl of ergosterol dissolved in 1:3 ether:hexane (0.002% w/v) was added to each plate. Solvent was allowed to evaporate prior to use.

Yeast cultivation for sterol production

Cultivation of strains for sterol production was performed in yeast extract peptone medium containing 80 g/l glucose. Yeast strains were inoculated into 2.5 ml YPD in 24-deepwell plates with air-penetrable lids (EnzyScreen, Netherlands). The plates were incubated at 30°C with 300 rpm agitation at 5 cm orbit cast for 24 hours. The cultures were then diluted to OD_{600} 0.1 in 2.5 ml fresh YPD-media with 80 g/l glucose and grown for a further 72 hours at 30°C with 300 rpm agitation. All cultivations were performed in triplicate. Cell dry weight was measured at the end of cultivation: 1 ml of culture broth was transferred into a pre-weighed 2 ml microcentrifuge tube, centrifuged (3000 g, 5 min) and

the supernatant was discarded. The cells were washed twice with deionized water (1 ml). The cell pellet was dried at 60°C for 7 days before the final weight was measured.

Chemicals were obtained, if not indicated otherwise, from Sigma-Aldrich. Nourseothricin was purchased from Jena BioScience GmbH (Germany).

Plasmid construction

The plasmids, BioBricks, and primers used in this study are listed in Tables 9-11, respectively.

Table 9: Plasmids for engineering *Yarrowia lipolytica* strains incapable of synthesising ergosterol

Plasmid name	Parent plasmid/ Reference	BioBricks
pCfB8861 (pHyg-YLgRNA3_IntE_4)	(Arnesen *et al*, 2020)	
pCfB6679 (pIntE_4-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB10252 (IntE4_PrTEF->DHCR7_Esilicul)	pCfB6679	BB3879:BB4879
pCfB10367 (Erg5_tPr48)		BB1135:BB5040:BB5101:BB5044
pCfB3405 (pORI1001-Nat-CEN1-USER)	(Holkenbrink *et al*, 2018)	
pCfB8843 (pORI1001-Hyg-CEN1-USER)	(Arnesen *et al*, 2020)	
pCfB10243 (pERG4_gRNA_KO_Hyg)	pCfB8843 (pORI1001-Hyg-CEN1-USER)	BB1635:BB1636:PR-27625:PR-27626
pCfB10244 (pERG5_gRNA_KO_Hyg)	pCFB8843 (pORI1001-Hyg-CEN1-USER)	BB1635:BB1636:PR-27627:PR-27628
pCfB10245 (pERG6_gRNA_KO_Hyg)	pCFB8843 (pORI1001-Hyg-CEN1-USER)	BB1635:BB1636:PR-27629:PR-27630
pCfB10246 (pERG4_gRNA_KO_Nat)	pCfB3405 (pORI1001-Nat-CEN1-USER)	BB1635:BB1636:PR-27625:PR-27626
pCfB10247 (pERG5_gRNA_KO_Nat)	pCfB3405 (pORI1001-Nat-CEN1-USER)	BB1635:BB1636:PR-27627:PR-27628
pCfB10248 (pERG6_gRNA_KO_Nat)	pCfB3405 (pORI1001-Nat-CEN1-USER)	BB1635:BB1636:PR-27629:PR-27630
pCfB10347 (pERG4_gRNA2_KO_Hyg)	pCFB8843 (pORI1001-Hyg-CEN1-USER)	BB1635:BB1636:PR-27929:PR-27930
pCfB10348 (pERG5_gRNA2_KO_Hyg)	pCFB8843 (pORI1001-Hyg-CEN1-USER)	BB1635:BB1636:PR-27931:PR-27932
pCfB10349 (pERG6_gRNA2_KO_Hyg)	pCFB8843 (pORI1001-Hyg-CEN1-USER)	BB1635:BB1636:PR-27933:PR-27934

pCfB10350 (pERG4_gRNA2_KO_Nat)	pCfB3405 (pORI1001-Nat-CEN1-USER)	BB1635:BB1636:PR-27929:PR-27930
pCfB10351 (pERG5_gRNA2_KO_Nat)	pCfB3405 (pORI1001-Nat-CEN1-USER)	BB1635:BB1636:PR-27931:PR-27932
pCfB10352 (pERG6_gRNA2_KO_Nat)	pCfB3405 (pORI1001-Nat-CEN1-USER)	BB1635:BB1636:PR-27933:PR-27934
pCfB5935 (pIntA-1-HphMx-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB4788 (pIntA_1-Nat-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6677 (pIntE_1-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB10494 (IntE3_PrGPAT->TtSTC)	pCfB6681	BB1617 (PrGPAT): BB5100 (TtSTC_USER)
pCfB10224 (ERG4_KORep_90bp)	DNA Duplex Synthesis from IDT (n.b. NOT a plasmid)	Sequence: ACATAAAGCCATCCACCCTTCCTTCGCAACCACACACACCATACAACGATAAGCTTAGTGAGCGAATGGTGAGGTTACTTAATTGAGTGG
pCfB10225 (ERG5_KORep_90bp)	DNA Duplex Synthesis from IDT (n.b. NOT a plasmid)	Sequence: AACTTCTCTCTCTCACACCACCACCACAACACAACTCCGCCCACCGGTATATAGGTTTGGTAATGTATTAATATTAATGATGGGGCGAGG
pCfB10226 (ERG6_KORep_90bp)	DNA Duplex Synthesis from IDT (n.b. NOT a plasmid)	Sequence: GATCTGAATCGCCCTTGTAAACCCCCCCAAAACACCACATTCAACACTGAGTAACTTATAGAGGGAGCCACGGCCCCAAAATTTATAATG

Table 10: Biobricks for engineering *Yarrowia lipolytica* strains incapable of synthesising ergosterol

BioBrick name	Template/Reference	Forward primer	Reverse primer
BB1635 (gRNA Pr)	(Holkenbrink *et al*, 2018)		
BB1636 (gRNA Ter)	(Holkenbrink *et al*, 2018)		
BB3879 (PrTefint->)	(Arnesen *et al*, 2020)		
BB4879 (PrTEF_DHCR7Es)	*Ectocarpus siliculosus* delta-7 sterol reductase codon optimised	PR-27571	PR-27572
BB5356 (Erg4_1kbUP_U2)	ST9100 gDNA	PR-27891	PR-27892
BB5092 (Erg4_1kbDOWN_U2)	ST9100 gDNA	PR-27893	PR-27894
BB5359 (Erg4_2kb_Rtemp)	BB5356 + BB5092	PR-27891	PR-27894
BB5357 (Erg5_1kbUP_U2)	ST9100 gDNA	PR-27895	PR-27896
BB5093 (Erg5_1kbDOWN_U2)	ST9100 gDNA	PR-27897	PR-27898

BB5360 (Erg5_2kb_Rtemp)	BB5357 + BB5093	PR-27895	PR-27898
BB5358 (Erg6_1kbUP_U2)	ST9100 gDNA	PR-27899	PR-27900
BB5094 (Erg6_1kbDOWN_U2)	ST9100 gDNA	PR-27901	PR-27902
BB5361 (Erg6_2kb_Rtemp)	BB5358 + BB5094	PR-27899	PR-27902
BB5093 (*ERG4*_1kbUP_U1)	ST9100 gDNA	PR-27891	PR-28358
BB5093 (*ERG5*_1kbUP_U1)	ST9100 gDNA	PR-27895	PR-28359
BB5093 (*ERG6*_1kbUP_U1)	ST9100 gDNA	PR-27899	PR-28360
BB5041 (LoxP_HygMX)	pCfB5935	PR-27972	PR-27973
BB5101 (LoxP_NatMX)	pCfB4788	PR-27972	PR-27973
BB5097 (Erg4_HphMX_KO)	BB5091 + BB5092 + BB5041	PR-27891	PR-27894
BB5098 (Erg5_HphMX_KO)	BB5093 + BB5094 + BB5041	PR-27895	PR-27898
BB5099 (Erg6_HphMX_KO)	BB5095 + BB5096 + BB5041	PR-27899	PR-27902
BB5246 (Erg4_NatMX_KO)	BB5091 + BB5092 + BB5101	PR-27891	PR-27894
BB5247 (Erg5_NatMX_KO)	BB5093 + BB5094 + BB5101	PR-27895	PR-27898
BB5248 (Erg6_NatMX_KO)	BB5095 + BB5096 + BB5101	PR-27899	PR-27902
BB5040 (Erg5_UPintergenic)	ST9100 gDNA	PR-27970	PR-27971
BB5044 (Erg5_tPr48_ORF996)	ST9100 gDNA	PR-27976	PR-27969
BB1135 (Easy Clone vector backbone)	pCfB6677	PR-11110	PR-11111
BB1617 (PrGPAT->)	(Holkenbrink *et al*, 2018)		
BB5100 (TtSTC_USER)	*Tetrahymena thermophilia* squalene-tetrahymanol cyclase codon optimised	PR-28361	PR-28362

Table 11: Primers for engineering *Yarrowia lipolytica* strains incapable of synthesising ergosterol

Primer name	**Sequence (5' → 3')**
PR-14617 (vector verification *E. coli* cPCR)	tatccctgtgttgaatc
PR-14619 (vector verification *E. coli* cPCR)	tatcgacccagttagc
PR-8859 (integration verification Yl cPCR)	aagtgtggatggggaagtgag
PR-14576 (IntE_3 verification Yl cPCR)	cacgcgautgaaggaaatgcctaaaacc
PR-14835 (IntE_3 verification Yl cPCR)	cacgcacgccattctataag
PR-14592 (IntE_4 verification Yl cPCR)	acgcgauttaacactggaccgtactgc

PR-20880 (IntE_4 verification YI cPCR)	attgctaagcgaccatagac
PR-27571 (A_Es_Fw)	acttttgcagtacuaaccgcagatcgacggcgctgccatcg
PR-27572 (A_Es_Rv)	cacgcgauttacaggatgccgggcacgatc
PR-27625 (ERG4_gRNA_Fw)	ggtctcgtactgcttgacagcgggttttagagct
PR-27626 (ERG4_gRNA_Rv)	ccgctgtcaagcagtacgagacctaaccaacct
PR-27627 (ERG5_gRNA_Fw)	gtagggaaccataatgacaggggttttagagct
PR-27628 (ERG5_gRNA_Rv)	cccctgtcattatggttccctactaaccaacct
PR-27629 (ERG6_gRNA_Fw)	agaaacaaacagcatcatgggggttttagagct
PR-27630 (ERG6_gRNA_Rv)	cccccatgatgctgtttgtttcttaaccaacct
PR-27891 (Erg4Rep_P1F)	ctctcaacaccttcaccgc
PR-27892 (Erg4Rep_P1R)	acctgcacuacgataagcttagtgagcg
PR-27893 (Erg4Rep_P2F)	gtgcaggutgtgtggttgcgaaggaag
PR-27894 (Erg4Rep_P2R)	gcactcaaaataccccgttc
PR-27895 (Erg5Rep_P1F)	ctcggtttgttgcagcagg
PR-27896 (Erg5Rep_P1R)	acctgcacuatggtccgtatcgtgaaatg
PR-27897 (Erg5Rep_P2F)	gtgcaggugggcggagttgtgttgtg
PR-27898 (Erg5Rep_P2R)	ggtcggctatccaatacatctc
PR-27899 (Erg6Rep_P1F)	gctacaagccggaggggaac
PR-27900 (Erg6Rep_P1R)	acctgcacugttgaatgtggtgttttggg
PR-27901 (Erg6Rep_P2F)	gtgcagguactgagtaacttatagaggg
PR-27902 (Erg6Rep_P2R)	ctgtaccgtttggaggactc
PR-27929 (ERG4_gRNA2_Fw)	atctcgtcgacctactacgagttttagagct
PR-27930 (ERG4_gRNA2_Rv)	tcgtagtaggtcgacgagattaaccaacct
PR-27931 (ERG5_gRNA2_Fw)	tgagaaatacaaggcccagtgttttagagct
PR-27932 (ERG5_gRNA2_Rv)	actgggccttgtatttctcataaccaacct
PR-27933 (ERG6_gRNA2_Fw)	ttcccgatactacaagggaggttttagagct
PR-27934 (ERG6_gRNA2_Rv)	ctcccttgtagtatcgggaataaccaacct
PR-27969 (Erg5ORF_R_U3)	cacgcgautcggtcggcaacaatctg
PR-27970 (Erg5_UP_uORF_F_U3)	cgtgcgautaagcatgcatcggacac
PR-27971 (Erg5_UP_uORF_R_U1)	atcgcacgugatcgtgtgagtcagagg
PR-27976 (ERG5_tPr50_F_U2)	agtgcaggucacaacttctctctctcacac
PR-27972 (Hyg/NatMX_F_U1)	cgtgcgautcagctgaagcttcgtac
PR-27973 (Hyg/NatMX_R_U2)	acctgcacugcataggccactagtgg
PR-28358 (Erg4Rep_P1R_U1)	atcgcacgugataagcttagtgagcgaatgg
PR-28359 (Erg5Rep_P1R_U1)	atcgcacgutggtccgtatcgtgaaatgg
PR-28360 (Erg6Rep_P1R_U1)	atcgcacgucaagggcgattcagatcagc
PR-27631 (ERG4_KOchk_Fw)	cctgatattggtgatcctcc
PR-27632 (ERG4_KOchk_Rv1)	agagccttgtttccgaggtg
PR-27633 (ERG4_KOchk_Rv2)	atacaatcccataggctggc
PR-11138 (HphMX_KOcasette_chkFw)	agcaatggguaaaaagcctgaactcaccgc
PR-28001 (Derg4_chkRv)	cgtgcgaugcttgccctggactacatcttg
PR-27634 (ERG5_KOchk_Fw)	acttctctctctcacaccacc
PR-27635 (ERG5_KOchk_Rv1)	ctgagggctctgttggtgaag
PR-27636 (ERG5_KOchk_Rv2)	accagtgtggttgtaaggatg
PR-22830 (HphMX_KOcasette_chkRv)	tcatactcaccgaaacgtg
PR-27970 (Derg5_chkRv)	cgtgcgautaagcatgcatcggacac
PR-27637 (ERG6_KOchk_Fw)	ctcgcatacttcccgtttgg
PR-27638 (ERG6_KOchk_Rv1)	tgagaccgacaatgttggc

PR-27639 (ERG6_KOchk_Rv2)	ccaccgatccttctcagctac
PR-26681 (NatMX_KOcasette_chkFw)	atccgctctaaccgaaaagg
PR-28007 (Derg6_chkFw)	cgtgcgaugaaggagatactggtgcc
PR-11110 (E.coli backboneUSER _fw)	atcgcgtgcattcgcggccgcatttaaatcc
PR-11111 (E.coli backboneUSER _rev)	atcgcacgcattcgcggccgcaaatttaaataaaatg
PR-28361 (TtSTC_F_U3)	atctgtcaugccacaatgaagaagatcctcatcggtc
PR-28362 (TtSTC_Rv)	cacgcgauttagatgttctgcttctggacg

BioBricks were again amplified by PCR. The PCR mix contained 32 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 1 µl 10mM dNTP, 1.5 µl $MgCl_2$, 2.5 µl 10 µM Fw primer, 2.5 µl 10 µM Rv primer, 0.2 µl Phusion® U polymerase and 10 ng DNA template. Reactions were multiplexed 8x per BioBrick. The cycling program was: 98°C for 1 min, 30 cycles of [98°C for 10 sec, gradient from 50 to 58°C for 20 sec, 72°C for 30 s/kb], 72°C for 5 min, pause at 10°C. The gene fragments were purified from agarose gels using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Biobricks were assembled into the EasyCloneYALI vectors with USER cloning (Holkenbrink *et al*, 2018). The USER reactions were transformed into *E. coli* and correct assemblies were verified by Sanger sequencing (Eurofins).

The delta-7 sterol reductase from *Ectocarpus siliculosus* (GenBank accession: CBN77313.1) and the squalene-tetrahymanol cyclase from *Tetrahymena thermophilia* (accession: XP_001026696.2) were codon-optimized for *Y. lipolytica* and synthesised as GeneArt String DNA fragments by ThermoFischer Scientific. The codon-optimized sequences are given in Table 4. 90 bp repair templates were synthesised as double-stranded DNA oligos (IDT DNA). Longer repair templates were generated using DNA fragments comprising upstream and downstream homology arms synthesis by PCR amplification from gDNA.

Yeast transformation

The yeast vectors and biobricks were transformed into *Y. lipolytica* using the lithium acetate transformation protocol as described by Holkenbrink *et al*, 2018. Vectors were digested prior to transformation with *Not*I enzyme (New England BioLabs) at 37°C for 1 hr and the digested product purified from solution using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Transformants were selected on antibiotic supplemented plates and correct transformants confirmed by colony PCR using Taq DNA Polymerase Master Mix RED (Ampliqon) with vector-specific primers and primers complementary to the genomic region adjacent to the integration site.

In summary, for marker-mediated gene deletion, *Y. lipolytica* strains were transformed with biobricks assembled by USER reaction as further detailed below. Transformants were selected on antibiotic supplemented plates and correct transformants confirmed by colony PCR. Marker removal was performed by transformation of the strains with a Cre-recombinase episomal vector. Marker removal was confirmed by colony PCR.

Results

Unsuccessful ERG4/ERG5/ERG6 knock-out attempts with guide RNA mediated gene deletion

Guide RNA (gRNA) vectors against ERG4 (YALI1_D24361g), ERG5 (YALI1_A18344g) and ERG6 (YALI1_F12138g) were assembled as described by Holkenbrink *et al*, 2018. For each of ERG4, ERG5 and ERG6, strain ST9100 was transformed with 500 ng gRNA (pCfB10243 to pCfB10245) and 2 nmole of the corresponding 90 bp repair template. Transformants were selected on YPD plates supplemented with hygromycin. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

The transformation was repeated with 1 μg gRNA vector and 4 nmole of the corresponding repair template. Transformants were selected on YPD plates supplemented with hygromycin and ergosterol. No colonies contained deletions in the relevant ERG genes.

Ectocarpus siliculosus delta-7 sterol reductase was integrated into the ST9100 genome under the control of the PrTEFintron promoter, generating strain ST10924. Strain ST10924 was transformed with 1 μg *ERG5* gRNA vector (pCfB10247) and 4 nmole of repair template. Transformants were selected on YPD plates supplemented with nourseothricin and ergosterol. No colonies contained deletions in the relevant ERG genes.

New repair templates were constructed comprising 1 kb homology arms either side of the ERG4/5/6 coding sequences, amplified from ST9100 gDNA (~ 2kb total length). ST9100 was transformed with 500 ng gRNA (pCfB10243 to pCfB10245) and 500 ng of the corresponding 2 kb repair template. Transformants were selected on YPD plates supplemented with hygromycin and ergosterol. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

Strain ST10924 was transformed with 500 ng ERG5 gRNA vector (pCfB10247) and 500ng of repair template. Transformants were selected on YPD plates supplemented with nourseothricin and ergosterol. No colonies were obtained.

For each of ERG4, ERG5 and ERG6, new gRNAs were designed using the best performing crRNA sequences as identified by Schwartz *et al.*, 2019. Strain ST9100 was transformed with 500 ng gRNA (pCfB10347 to pCfB10352) and 500 ng of the corresponding 2 kb repair template. Transformants were selected on YPD plates supplemented with hygromycin and ergosterol. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

Strains ST9100 and ST10924 were transformed with 1 μg ERG5 gRNA (pCfB10244 to pCfB10348) and 500 ng of the corresponding 2 kb repair template. Transformants were selected on YPD plates supplemented with hygromycin and ergosterol. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

Resistance marker-mediated gene deletion

New ERG4/5/6 repair templates were constructed for marker-mediated knock-out, comprising 1 kb homology arms either side of a hygromycin (HphMX) or nourseothricin (NatMX) resistance marker. Strains ST9100 and ST10924 were transformed with 5 μg of the *ERG5* HphMX cassette.

Transformants were selected on YPD plates supplemented with hygromycin and YPD plates supplemented with hygromycin and ergosterol. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

Strains ST9100, ST4842 and ST4840 were transformed with 2 μg of the ERG5 HphMX cassette. Transformants were selected on YPD plates supplemented with hygromycin and YPD plates supplemented with hygromycin and ergosterol. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

Strains ST9100 and ST4842 were transformed with 5 μg of the ERG4 NatMX cassette. Transformants were selected on YPD plates supplemented with hygromycin and YPD plates supplemented with hygromycin and ergosterol. Gene deletion was examined by colony PCR; no colonies contained deletions in the relevant ERG genes.

The ERG5 promoter was truncated to 48 bp to down-regulate ERG5 expression. A cassette was generated comprising a NatMX resistance marker flanked by two homology arms: the region between 2.5 and 1.5 kb upstream of the ERG5 coding sequence, and the region from 48 bp upstream of the start codon plus 996 bp of the ERG5 open reading frame. The cassette was assembled into a plasmid and transformed into ST10924 to generate ST10934. ERG5 promoter truncation was confirmed by sequencing. Strain ST10934 was transformed with 7.5 μg of either the ERG4 or ERG6 HphMX cassettes. Transformants were selected on YPD plates supplemented with hygromycin. No colonies were obtained.

Efficient ERG4/ERG5/ERG6 knock-out with intracellular provision of tetrahymanol

The gene encoding squalene-tetrahymanol cyclase from *Tetrahymena thermophilia* (*TtSTC*) was integrated into the ST9100 platform strain genome under the control of the Pr*GPAT* promoter, previously characterised as a weak promoter (Holkenbrink *et al*, 2018). This promoter was selected to drive minimal expression of *TtSTC* to limit diversion of carbon flux away from the main sterol pathway. [Note: the promoter described as 'PrGPAT' in Holkenbrink *et al*. 2018 and used in this study does not belong to the *Y. lipolytica GPAT* gene (YALI1_C00230g, (Magnan et al. 2016), but is instead the sequence corresponding to the promoter region of gene YALI1_C00209g in *Y. lipolytica* W29 Y-63746 with genomic location of the promoter being Chromosome 1C:20927-22056, and genomic location of the downstream gene being Chromosome 1C: 18,422-20,926.] The resulting strain ST11005 produced 0.4 mg/g dry cell weight (DCW) tetrahymanol, comprising 14.0% of the total sterol fraction.

Construction of *Yarrowia lipolytica* strains incapable of synthesizing ergosterol

The tetrahymanol producing strain ST11005 was used for the construction of strains incapable of synthesising ergosterol. Firstly, to create a ΔERG5 strain, the ERG5 gene (sterol C-22 desaturase, YALI1_A18344g) was deleted by marker-mediated knock out. A knock-out cassette was constructed comprising a hygromycin resistance marker (HphMX) flanked by 1 kb homology arms corresponding to 1 kb upstream and downstream of the ERG5 coding sequence. This construct was used to transform ST11005 thereby generating ST11014.

ST11005 was transformed with 3 μg of the ERG5 HphMX cassette. Transformants were selected on YPD plates supplemented with hygromycin. Gene deletion was examined by colony PCR; all colonies tested contained deletion of *ERG5* (ST11014).

The HphMX resistance marker was subsequently looped out by Cre-lox recombination. The resulting strain ST11027 produced 3.1 mg/g DCW ergosta-5,7-dienol, comprising 60.0% of the total sterol fraction. The strain also produced 5.2 mg/g DCW tetrahymanol, comprising 36.7% of the total sterol fraction.

To create a ΔERG4ΔERG5 strain, the ERG4 gene (delta-24 sterol reductase, YALI1_D24361g) was deleted by HphMX marker-mediated knockout in an analogous manner. The resulting strain ST11040 produced 3.1 mg/g DCW ergosta-5,7,24(28)-trienol, comprising 20.0% of the total sterol fraction. The strain also produced 15.8 mg/g DCW tetrahymanol, comprising 75.2% of the total sterol fraction.

Alternatively, strain ST11014 was transformed with 4 μg of the ERG4 NatMX cassette. Transformants were selected on YPD plates supplemented with nourseothricin. Gene deletion was examined by colony PCR; all colonies tested contained deletion of ERG4 (ST11028).

To create a ΔERG5ΔERG6 strain, the starting yeast strain was ST11027 (the strain obtained following the HphMX resistance marker in the *ERG5* locus of ST11014 being looped out by Cre-Lox recombination).The gene encoding *ERG6* (sterol methyl transferase, YALI1_F12138g) was deleted from ST11027 by HphMX marker-mediated knockout in an analogous manner, generating strain ST11325. The HphMX resistance marker was subsequently looped out by Cre-Lox recombination. The resulting strain ST11330 produced 0.2 mg/g DCW zymosterol, comprising 0.6% of the total sterol fraction. The strain also produced 36.0 mg/g DCW tetrahymanol, comprising 99.4% of the total sterol fraction.

Strain ST11027 was transformed with 2 μg of the ERG6 HphMX cassette. Transformants were selected on YPD plates supplemented with hygromycin. Gene deletion was examined by colony PCR; most colonies tested contained deletion of ERG6 (ST11325).

Compared to the wild-type strains ST4840 and ST4842, strain ST6512 is optimised for greater efficiency of homologous recombination. ST6512 was transformed with 8 μg of either the ERG4 or ERG5 NatMX cassettes. Transformants were selected on YPD plates supplemented with nourseothricin. Gene deletion was examined by colony PCR. In each case, only one of the colonies tested contained deletion of the relevant gene (ST11326 and ST11327).

Discussion

Strains used in this work derived from *Y. lipolytica* W29 strain Y-63746 (MatA, ARS Culture Collection, NCAUR, United States, ATCC20460™). Strains used in prior work included the E122-derived strain ATCC 201249 (MATA ura3-302 leu2-270 lys8-11 PEX17-HA (Du *et al*., 2016 & Zhang *et al* . 2017) and W29-derived strain Po1f (ATCC® MYA-2613™, MATA ura3-302 leu2-270 xpr2-322 axp2-deltaNU49 XPR2::SUC2). It is possible that these different strain backgrounds alter the essentiality of ERG5. ERG5 was identified as non-essential by transposon mutagenesis in strain W29 (CLIB89/ ATCC20460™, Patterson et al., 2018) and CRISPR-Cas9 mediated gene disruption in PO1f (MatA,

leu2-270, ura3- 302,xpr2-322, axp-2, Schwartz et al., 2019). ERG4 was identified as essential by Patterson et al., 2018, but results were inconclusive for Schwartz et al, 2019. ERG6 was classed as essential by both studies. (It is noted that the method employed by Schwartz et al., 2019, also classified genes such as ERG25, ERG26 and ERG8 as non-essential, although these are essential in *Saccharomyces cerevisiae.* This is unexpected as *Yarrowia* lacks homologues of the sterol uptake transporters found in Saccharomyces yeasts which allow certain ERG genes to be knocked out when ergosterol is provided in the growth medium).

The platform strain ST9100 is modified for increased squalene synthesis, the precursor to the sterol pathway. This suggests that the sterol pathway may be carefully regulated at multiple levels to prevent build-up of ergosterol. Regulatory mechanisms may include end-product feedback inhibition by ergosterol. This mechanism may be highly specific for ergosterol, to prevent feedback inhibition by structurally similar early intermediates of the sterol pathway. Whilst ergosterol production by this strain is not greater than the unmodified base strain, strains derived from ST9100 that are incapable of ergosterol production show significantly increased sterol titre. For example, flux through the sterol pathway is greater in ST11027 (ΔERG5, total sterol: 5.2 mg/g dry cell weight (DCW) and ST11040 (ΔERG5ΔERG4, total sterol: 15.8 mg/g DCW) than ST9100 (total sterol: 2.1 mg/g DCW). ERG4/5 deletion in a platform strain may therefore be more harmful to the cell due to the accumulation of sterol intermediates which may be toxic in high concentrations, for example due to detrimental effects to the membrane.

The higher efficiency of ERG4/5/6 knock-out in strains capable of synthesising tetrahymanol may indicate that tetrahymanol is able to substitute for ergosterol function in the cell. This may mitigate the detrimental effects of accumulation of sterol intermediates upon ERG gene deletion. Overall, the ergosterol biosynthesis pathway is highly demanding (especially due to oxygen and NADH requirements). However, the prevalence of ergosterol as the dominant sterol across yeast species suggests that ergosterol is superior to the preceding intermediates for cellular function, despite high structural similarity. This may explain the difficulty in obtaining strains incapable of producing ergosterol, in the absence of a suitable surrogate.

Additional References:

Du HX, Xiao WH, Wang Y, Zhou X, Zhang Y, Liu D & Yuan YJ (2016) Engineering Yarrowia lipolytica for campesterol overproduction. *PLoS One*

Magnan C, Yu J, Chang I, Jahn E, Kanomata Y, Wu J, Zeller M, Oakes M, Baldi P & Sandmeyer S (2016) Sequence assembly of Yarrowia lipolytica strain W29/CLIB89 shows transposable element diversity. *PLoS One* 11

Patterson K, Yu J, Landberg J, Chang I, Shavarebi F, Bilanchone V & Sandmeyer S (2018) Functional genomics for the oleaginous yeast Yarrowia lipolytica. *Metab. Eng.* **48:** 184–196

Schwartz C, Cheng JF, Evans R, Schwartz CA, Wagner JM, Anglin S, Beitz A, Pan W, Lonardi S, Blenner M, Alper HS, Yoshikuni Y & Wheeldon I (2019) Validating genome-wide CRISPR-Cas9 function improves screening in the oleaginous yeast Yarrowia lipolytica. *Metab. Eng.* **55:** 102–110

Zhang Y, Wang Y, Yao M, Liu H, Zhou X, Xiao W & Yuan Y (2017) Improved campesterol production in engineered Yarrowia lipolytica strains. *Biotechnol. Lett.*

Example 3 - Engineering of *Yarrowia lipolytica* for production of non-native sterols

Materials and Methods

Strains, culture conditions and chemicals

Escherichia coli strain DH5α was used for plasmid construction as described above in Example 1.

The *Y. lipolytica* strain ST9100 derived from the *Y. lipolytica* W29 strain Y-63746 (Mat A, ATCC20460™/ ARS Culture Collection,NCAUR, United States) as described above was used to construct all non-native sterol-producing strains. All strains are detailed in Table 5.

Y. lipolytica was grown at 30°C on yeast extract peptone dextrose (YPD) media containing 10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose, supplemented with 20 g/l agar for preparation of solid media. For selection, either nourseothricin (250 mg/l) or hygromycin (400 mg/l) was added to the media.

Cultivation of strains for sterol production was performed in yeast extract peptone medium containing 80 g/l glucose as described more fully above.

Chemicals were obtained, if not indicated otherwise, from Sigma-Aldrich. Nourseothricin was purchased from Jena BioScience GmbH (Germany).

Plasmid construction

Delta-7 sterol reductase sequences from *Solanum tuberosum*, (GenBank accession: BAQ55276.1), *Danio rerio* (accession: NP_958487.2), *Legionella drancourtii* (accession: FJ197317.1), *Ectocarpus siliculosus* (accession: CBN77313.1), *Candidatus Protochlamydia amoebophilia* (accession: KIC71363.1), *Coccomyxa subellipsoidea* (accession: XM_005650286.1), *Mortierella vertcillata* (accession: KFH65691.1), *Glycine soja* (accession: XP_028244742.1); Tetraselmis sp. GSL018 (accession: JAC78771.1), and *Waddlia chondrophila* (accession: ADI39181.1), and squalene-tetrahymanol cyclase from *Tetrahymena thermophilia* (accession: XP_001026696.2) were codon-optimized for *Y. lipolytica* and the codon-optimised sequences synthesised as GeneArt String DNA fragments by ThermoFischer Scientific. The codon-optimized sequences are listed in Table 4a.

Delta-24 sterol reductase sequences from *Danio rerio* (accession: BC086711.1) and *Mus musculus* (accession: NM_053272.2) plus C-28 sterol methyl transferase sequences from *Arabidopsis thaliana* (accession: NM_101884.4), *Amborella trichopoda* (accession: XP_006828830.1) and *Chenopodium quinoa* (accession: XP_021737090.1) were codon-optimised for *Y. lipolytica* (see Tables 4a and 4b). The codon-optimised sequences were again synthesised as above.

The plasmids, biobricks, and primers used in this study are listed in Tables 12-14, respectively. BioBricks were amplified by PCR. The PCR mix contained 32 μl water, 10 μl high fidelity Phusion® polymerase buffer (5x), 1 μl 10mM dNTP, 1.5 μl $MgCl_2$, 2.5 μl 10 μM Fw primer, 2.5 μl 10 μM Rv primer, 0.2 μl Phusion® U polymerase, and 10 ng DNA template. Reactions were multiplexed 8x per BioBrick. The cycling program was: 98°C for 1 min, 30 cycles of [98°C for 10 sec, gradient from 50 to 58°C for 20 sec, 72 °C for 30 s/kb], 72°C for 5 min, pause at 10°C. The gene fragments were purified

from agarose gels using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Biobricks were assembled into the EasyCloneYALI vectors with USER cloning (Holkenbrink et al. 2018). The USER reactions were transformed into *E. coli* and correct assemblies were verified by Sanger sequencing (Eurofins).

Table 12a: Plasmids for engineering of *Yarrowia lipolytica* for production of non-native sterols

Plasmid name	Parent plasmid/ Reference	BioBricks
pCfB3405 (pORI1001-Nat-CEN1-USER)	(Holkenbrink *et al*, 2018)	
pCfB4788 (pIntA_1-Nat-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB5935 (pIntA-1-HphMx-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCFB8843 (pORI1001-Hyg-CEN1-USER)	(Arnesen *et al*, 2020)	
pCfB6637 (pNat-YLgRNA3_IntE_3)	(Holkenbrink *et al*, 2018)	
pCfB6638 (pNat-YLgRNA2_IntE_4)	(Holkenbrink *et al*, 2018)	
pCfB6677 (pIntE_1-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6679 (pIntE_4-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB8861 (pHyg-YLgRNA3_IntE_4)	(Arnesen *et al*, 2020)	
pCfB6681 (pIntE_3-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6611 (pNat-PrExp-Cre)	pCfB4158 (pPrExp-Cre) - (Holkenbrink *et al*, 2018)	
pCfB10249 (IntE4_PrTEF->DHCR7_Stuberosum)	pCfB6679	BB3879 (Tefint->):BB4876 (PrTEF_DHCR7St)
pCfB10250 (IntE4_PrTEF->DHCR7_Drerio)	pCfB6679	BB3879 (Tefint->):BB4877 (PrTEF_DHCR7Dr)
pCfB10251 (IntE4_PrTEF->DHCR7_Ldrancou)	pCfB6679	BB3879 (Tefint->):BB4878 (PrTEF_DHCR7Ld)
pCfB10252 (IntE4_PrTEF->DHCR7_Esilicul)	pCfB6679	BB3879 (Tefint->):BB4879 (PrTEF_DHCR7Es)
pCfB10253 (IntE4_PrTEF->DHCR7_Cprotoch)	pCfB6679	BB3879 (Tefint->):BB4880 (PrTEF_DHCR7Cp)
pCfB10254 (IntE4_PrTEF->DHCR7_Csubellip)	pCfB6679	BB3879 (Tefint->):BB4881 (PrTEF_DHCR7Cs)
pCfB10255 (IntE4_PrTEF->DHCR7_Mverticillata)	pCfB6679	BB3879 (Tefint->):BB4882 (PrTEF_DHCR7Mv)
pCfB10256 (IntE4_PrTEF->DHCR7_Gsoja)	pCfB6679	BB3879 (Tefint->):BB4883 (PrTEF_DHCR7Gs)
pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)	pCfB6679	BB3879 (Tefint->):BB4884 (PrTEF_DHCR7Ts)
pCfB10258 (IntE4_PrTEF->DHCR7_Wchondrophi)	pCfB6679	BB3879 (Tefint->):BB4885 (PrTEF_DHCR7Wc)

pCfB10494 (IntE3_PrGPAT->TtSTC)	pCfB6681	BB1617 (PrGPAT): BB5100 (TtSTC_USER)
pCfB10367 (Erg5_tPr48)		BB1135:BB5040:BB5101:BB5044
pCfB7254 (gRNA for PAH1 deletion Y.L)	pCfB3405	BB1635: BB1636:PR-29832-PR29833

Table 12b: Plasmids for engineering of *Yarrowia lipolytica* for production of non-native sterols

Plasmid name	Parent plasmid/ Reference	BioBricks
pCfB6633 (pNat-YLgRNA2_IntE_1)	(Holkenbrink *et al*, 2018)	
pCfB6637 (pNat-YLgRNA3_IntE_3)	(Holkenbrink *et al*, 2018)	
pCfB6638 (pNat-YLgRNA2_IntE_4)	(Holkenbrink *et al*, 2018)	
pCfB6677 (pIntE_1-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6681 (pIntE_3-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB4783 (pIntE_3-Nat-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6679 (pIntE_4-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pBP8009 (pIntF_3-TPex20-TLip2)	Dr. K.R. Kildegaard, Biophero ApS, Denmark	
pBP8003 (pNat-YLgRNA4-IntF_3)	Dr. K.R. Kildegaard, Biophero ApS, Denmark	
pCfB8861 (pHyg-YLgRNA3_IntE_4)	(Arnesen *et al*, 2020)	
pCfB6611 (pNat-PrExp-Cre)	pCfB4158 (pPrExp-Cre) - (Holkenbrink *et al*, 2018)	
pCfB10251 (IntE4_PrTEF->DHCR7_Ldrancou)	pCfB6679	BB3879 (Tefint->):BB4878 (PrTEF_DHCR7Ld)
pCfB10254 (IntE4_PrTEF->DHCR7_Csubellip)	pCfB6679	BB3879 (Tefint->):BB4881 (PrTEF_DHCR7Cs)
pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)	pCfB6679	BB3879 (Tefint->):BB4884 (PrTEF_DHCR7Ts)
pCfB10293 (IntE1_PrTEF->DHCR24_Drerio)	pCfB6677	BB3879 (Tefint->):BB4965 (PrTEF_DHCR24Dr)
pCfB10298 (IntE1_PrTEF->DHCR24_Mmusculus)	pCfB6677	BB3879 (Tefint->):BB4970 (PrTEF_DHCR24Mm)
pCfB10311 (IntE1_PrTEF->SMT2_Cquinoa)	pCfB6677	BB3879 (Tefint->):BB4983 (PrTEF_SMT2Cq)
pCfB11486 (IntF3_SMT_Ath<-PrGPD_TEFi->SMT_Atr)	pBP8009	BB4900 (PrGPD_SMT2Atr): BB3865 (<-PrGPD_Tefint->): BB4977 (PrTEF_SMT2Ath)
pCfB10494 (IntE3_PrGPAT->TtSTC)	pCfB6681	BB1617 (PrGPAT): BB5100 (TtSTC_USER)

Table 13a: Biobricks for engineering of *Yarrowia lipolytica* for production of non-native sterols

BioBrick name	Template/Reference	Forward primer	Reverse primer
BB1635	(Holkenbrink *et al*, 2018)		
BB1636	(Holkenbrink *et al*, 2018)		
BB1617 (PrGPAT->)	(Holkenbrink *et al*, 2018)		
BB3879 (PrTefint->)	(Arnesen *et al*, 2020)		
BB4876 (PrTEF_DHCR7St)	*Solanum tuberosum* delta-7 sterol reductase codon optimised	PR-27565	PR-27566
BB4877 (PrTEF_DHCR7Dr)	*Danio rerio* delta-7 sterol reductase codon optimised	PR-27567	PR-27568
BB4878 (PrTEF_DHCR7Ld)	*Legionella drancourtii* delta-7 sterol reductase codon optimised	PR-27569	PR-27560
BB4879 (PrTEF_DHCR7Es)	*Ectocarpus siliculosus* delta-7 sterol reductase codon optimised	PR-27571	PR-27572
BB4880 (PrTEF_DHCR7Cp)	Candidatus *Protochlamydia amoebophila* delta-7 sterol reductase codon optimised	PR-27573	PR-27574
BB4881 (PrTEF_DHCR7Cs)	*Coccomyxa subellipsoidea* delta-7 sterol reductase codon optimised	PR-27575	PR-27576
BB4882 (PrTEF_DHCR7Mv)	*Mortierella verticillate* delta-7 sterol reductase codon optimised	PR-27577	PR-27578
BB4883 (PrTEF_DHCR7Gs)	*Glycine soja* delta-7 sterol reductase codon optimised	PR-27579	PR-27570
BB4884 (PrTEF_DHCR7Ts)	*Tetraselmis* sp. GSL018 delta-7 sterol reductase codon optimised	PR-27581	PR-27582
BB4885 (PrTEF_DHCR7Wc)	*Waddlia chondrophila* delta-7 sterol reductase codon optimised	PR-27583	PR-27584
BB5041 (LoxP_HphMX)	pCfB5935 - (Holkenbrink et al, 2018)	PR-27972	PR-27973
BB5101 (LoxP_NatMX)	pCfB4788	PR-27972	PR-27973
BB5091 (*erg4*_1kbUP_U1)	ST9100 gDNA	PR-27891	PR-28358
BB5092 (*erg4*_1kbDOWN_U2)	ST9100 gDNA	PR-27893	PR-27894
BB5093 (*erg5*_1kbUP_U1)	ST9100 gDNA	PR-27895	PR-28359
BB5094 (*erg5*_1kbDOWN_U2)	ST9100 gDNA	PR-27897	PR-27898
BB5095 (*erg6*_1kbUP_U1)	ST9100 gDNA	PR-27899	PR-28360
BB5096 (*erg6*_1kbDOWN_U2)	ST9100 gDNA	PR-27901	PR-27902
BB5097 (*erg4*_HphMX_KO)	BB5091 + BB5092 + BB5041 USER reaction	PR-27891	PR-27894
BB5098 (*erg5*_HphMX_KO)	BB5093 + BB5094 + BB5041 USER reaction	PR-27895	PR-27898
BB5099 (*erg6*_HphMX_KO)	BB5095 + BB5096 + BB5041 USER reaction	PR-27899	PR-27902
BB5100 (TtSTC_USER)	*Tetrahymena thermophilia* squalene-tetrahymanol cyclase codon optimised	PR-28361	PR-28362
BB5040 (Erg5_UPintergenic)	ST9100 gDNA	PR-27970	PR-27971
BB5044 (Erg5_tPr48_ORF996)	ST9100 gDNA	PR-27976	PR-27969

BB1135 (Easy Clone vector backbone)	pCfB6677	PR-11110	PR-11111
BB5312 (YlPAH1_repair-up)	ST9100 gDNA	PR-26303	PR-26304
BB5313 (YlPAH1_repair-dw)	ST9100 gDNA	PR-26305	PR-26306
BB5314 (YlPAH1_repair)	BB5312 + BB5313 USER ligation	PR-26303	PR-26306

Table 13b: Biobricks for engineering of *Yarrowia lipolytica* for production of non-native sterols

BioBrick name	Template/Reference	Forward primer	Reverse primer
BB1616 (PrDGA1->)	(Holkenbrink *et al*, 2018)		
BB1617 (PrGPAT->)	(Holkenbrink *et al*, 2018)		
BB3865 (<-PrGPD_Tefint->)	(Arnesen *et al*, 2020)		
BB3879 (PrTefint->)	(Arnesen *et al*, 2020)		
BB5041 (LoxP_HphMX)	pCfB5935 - (Holkenbrink et al, 2018)	PR-27972	PR-27973
BB5101 (LoxP_NatMX)	pCfB4788	PR-27972	PR-27973
BB5091 (*erg4*_1kbUP_U1)	ST9100 gDNA	PR-27891	PR-28358
BB5092 (*erg4*_1kbDOWN_U2)	ST9100 gDNA	PR-27893	PR-27894
BB5093 (*erg5*_1kbUP_U1)	ST9100 gDNA	PR-27895	PR-28359
BB5094 (*erg5*_1kbDOWN_U2)	ST9100 gDNA	PR-27897	PR-27898
BB5095 (*erg6*_1kbUP_U1)	ST9100 gDNA	PR-27899	PR-28360
BB5096 (*erg6*_1kbDOWN_U2)	ST9100 gDNA	PR-27901	PR-27902
BB5097 (*erg4*_HphMX_KO)	BB5091 + BB5092 + BB5041 USER reaction	PR-27891	PR-27894
BB5098 (*erg5*_HphMX_KO)	BB5093 + BB5094 + BB5041 USER reaction	PR-27895	PR-27898
BB5099 (*erg6*_HphMX_KO)	BB5095 + BB5096 + BB5041 USER reaction	PR-27899	PR-27902
BB5100 (TtSTC_USER)	*Tetrahymena thermophilia* squalene-tetrahymanol cyclase codon optimised	PR-28361	PR-28362
BB1135 (Easy Clone vector backbone)	pCfB6677	PR-11110	PR-11111
BB4878 (PrTEF_DHCR7Ld)	*Legionella drancourtii* delta-7 sterol reductase codon optimised	PR-27569	PR-27570
BB4881 (PrTEF_DHCR7Cs)	*Coccomyxa subellipsoidea* delta-7 sterol reductase codon optimised	PR-27575	PR-27576
BB4884 (PrTEF_DHCR7Ts)	*Tetraselmis* sp. GSL018 delta-7 sterol reductase codon optimised	PR-27581	PR-27582
BB4965 (PrTEF_DHCR24Dr)	*Danio rerio* delta-24 sterol reductase codon optimised	PR-27749	PR-27750
BB4970 (PrTEF_DHCR24Mm)	*Mus musculus* delta-24 sterol reductase codon optimised	PR-27759	PR-27760
BB4983 (PrTEF_SMT2Cq)	*Chenopodium quinoa* sterol methyltransferase codon optimised	PR-27785	PR-27786

BB4900 (PrGPD_SMT2Atr)	*Amborella trichopoda* sterol methyltransferase codon optimised	PR-27613	PR-27614
BB4977 (PrTEF_SMT2Ath)	*Arabidopsis thaliana* sterol methyltransferase codon optimised	PR-27773	PR-27774

Table 14a: Primers for engineering of *Yarrowia lipolytica* for production of non-native sterols

Primer name	Sequence (5' → 3')
PR-14617 (vector verification *E. coli* cPCR)	Tatccctgtgttgaatc
PR-14619 (vector verification *E. coli* cPCR)	Tatcgacccagttagc
PR-8859 (integration verification Yl cPCR)	Aagtgtggatggggaagtgag
PR-14576 (IntE_3 verification Yl cPCR)	Cacgcgautgaaggaaatgcctaaaacc
PR-14835 (IntE_3 verification Yl cPCR)	Cacgcacgccattctataag
PR-14592 (IntE_4 verification Yl cPCR)	Acgcgauttaacactggaccgtactgc
PR-20880 (IntE_4 verification Yl cPCR)	Attgctaagcgaccatagac
PR-11110 (E.coli backboneUSER _fw)	Atcgcgtgcattcgcggccgcatttaaatcc
PR-11111 (E.coli backboneUSER _rev)	Atcgcacgcattcgcggccgcaaatttaaataaaatg
PR-27565 (A_St_Fw)	Acttttgcagtacuaaccgcaggccgagtctcagctggtgcac
PR-27566 (A_St_Rv)	Cacgcgauttagtagatgccggggatcac
PR-27567 (A_Dr_Fw)	Acttttgcagtacuaaccgcagatggcctctgaccgagttcg
PR-27568 (A_Dr_Rv)	Cacgcgauttagaagatgttgggcagcag
PR-27569 (A_Ld_Fw)	Acttttgcagtacuaaccgcagtacttcaagatccgaaacac
PR-27570 (A_Ld_Rv)	Cacgcgauttagatcacgaagggcacgatc
PR-27571 (A_Es_Fw)	Acttttgcagtacuaaccgcagatcgacggcgctgccatcg
PR-27572 (A_Es_Rv)	Cacgcgauttacaggatgccgggcacgatc
PR-27573 (A_Cp_Fw)	Acttttgcagtacuaaccgcagctgatcgagatgctgtgcatc
PR-27574 (A_Cp_Rv)	Cacgcgauttagatcacgaaggggatgatc
PR-27575 (A_Cs_Fw)	Acttttgcagtacuaaccgcaggtcacaacccgagccgctg
PR-27576 (A_Cs_Rv)	Cacgcgauttagaaaatgtaagggatcatc
PR-27577 (A_Mv_Fw)	Acttttgcagtacuaaccgcaggccgtgcagcagcgaaagac
PR-27578 (A_Mv_Rv)	Cacgcgauttagtacacgtaggggatcagc
PR-27579 (A_Gs_Fw)	Acttttgcagtacuaaccgcagggcgctaccgtgcactctc
PR-27580 (A_Gs_Rv)	Cacgcgauttagtagatgccggggatg
PR-27581 (A_Ts_Fw)	Acttttgcagtacuaaccgcagaagcgagcctccaagaccc
PR-27582 (A_Ts_Rv)	Cacgcgauttagaagatgtagggcacgatc
PR-27583 (A_Wc_Fw)	Acttttgcagtacuaaccgcaggccgccaccaccaccaacg
PR-27584 (A_Wc_Rv)	Cacgcgauttagtagatgccggggatcag
PR-27891 (Erg4Rep_P1F)	Ctctcaacaccttcaccgc
PR-28358 (Erg4Rep_P1R_U1)	Atcgcacgugataagcttagtgagcgaatgg
PR-27893 (Erg4Rep_P2F)	gtgcaggutgtgtggttgcgaaggaag
PR-27894 (Erg4Rep_P2R)	gcactcaaaataccccgttc
PR-27895 (Erg5Rep_P1F)	ctcggtttgttgcagcagg
PR-28359 (Erg5Rep_P1R_U1)	atcgcacgutggtccgtatcgtgaaatgg
PR-27897 (Erg5Rep_P2F)	gtgcaggugggcggagttgtgttgtg

PR-27898 (Erg5Rep_P2R)	ggtcggctatccaatacatctc
PR-27899 (Erg6Rep_P1F)	gctacaagccggaggggaac
PR-28360 (Erg6Rep_P1R_U1)	atcgcacgucaagggcgattcagatcagc
PR-27901 (Erg6Rep_P2F)	gtgcagguactgagtaacttatagaggg
PR-27902 (Erg6Rep_P2R)	ctgtaccgtttggaggactc
PR-27972 (HphMX_F_U1)	cgtgcgautcagctgaagcttcgtac
PR-27973 (HphMX_R_U2)	acctgcacugcataggccactagtgg
PR-28361 (TtSTC_F_U3)	atctgtcaugccacaatgaagaagatcctcatcggtc
PR-28362 (TtSTC_Rv)	cacgcgauttagatgttctgcttctggacg
PR-22830 (□erg5_chk)	tcatactcaccgaaacgtg
PR-27961 (□erg5_chk)	gttccaatgcctggcaag
PR-27634 (□erg5_chk)	acttctctctctcacaccacc
PR-27635 (□erg5_chk)	ctgagggctctgttggtgaag
PR-27636 (□erg5_chk)	accagtgtggttgtaaggatg
PR-11138 (□erg4_chk)	agcaatggguaaaaagcctgaactcaccgc
PR-27631 (□erg4_chk)	cctgatattggtgatcctcc
PR-27632 (□erg4_chk)	agagccttgtttccgaggtg
PR-27633 (□erg4_chk)	atacaatcccataggctggc
PR-28001 (□erg4_chk)	cgtgcgaugcttgccctggactacatcttg
PR-22830 (□erg6_chk)	tcatactcaccgaaacgtg
PR-28007 (□erg6_chk)	cgtgcgaugaaggagatactggtgcc
PR-27637 (□erg6_chk)	ctcgcatacttcccgtttgg
PR-27639 (□erg6_chk)	ccaccgatccttctcagctac
PR-27969 (Erg5ORF_R_U3)	cacgcgautcggtcggcaacaatctg
PR-27970 (Erg5_UP_uORF_F_U3)	cgtgcgautaagcatgcatcggacac
PR-27971 (Erg5_UP_uORF_R_U1)	atcgcacgugatcgtgtgagtcagagg
PR-27976 (ERG5_tPr50_F_U2)	agtgcaggucacaacttctctctcacac
PR-26303 (YlPAH1_repair-up_fw)	acgtactgcgcccatatt
PR-26304 (YlPAH1_repair-up_rv)	aggccacutgtggttgtgtggtgatga
PR-26305 (YlPAH1_repair-dw_fw)	agtggccuattccagcccgtttcgt
PR-26306 (YlPAH1_repair-dw_rv)	cttggctttctagcggga
PR-26307 (YlPAH1_check_fw)	cgaacccaaatccggac
PR-26308 (YlPAH1_check_rv)	acctgctcctccaccta
PR-29832 (PAH1_gRNA_Sense)	ggaaggttagaagaagggaggttttagagct
PR-29833 (PAH1_gRNA_AntiSense)	ctcccttcttctaaccttcctaaccaacct

Table 14b: Additional primers for engineering of *Yarrowia lipolytica* for production of non-native sterols

Primer name	**Sequence (5' → 3')**
PR-14442 (IntE_1 verification Yl cPCR)	agttgtgaccaagacaaatg
PR-14398 (IntE_1 verification Yl cPCR)	cacgcgaUgttagaagcaattggagaag
PR-14837 (IntF_3 verification Yl cPCR)	acatgctcgcgcctcgatag

PR-14584 (IntF_3 verificiationYl cPCR)	cacgcgautttggtcgtcgcccaacaag
PR-27749 (B_Dr_Fw)	acttttgcagtacuaaccgcaggaccctctgctgtac
PR-27750 (B_Dr_Rv)	cacgcgauttagtgtcgggcagacttg
PR-27759 (B_Mm_Fw)	acttttgcagtacuaaccgcaggaacccgccgtgtctc
PR-27760 (B_Mm_Rv)	cacgcgauttagtgtcgagcggccttg
PR-27785 (C_Cq_Fw)	acttttgcagtacuaaccgcaggactctatggccctg
PR-27786 (C_Cq_Rv)	cacgcgauttaagagtcagacttggc
PR-27613 (C_At_Fw)	atctgtcaugccacaatggagactctggccgc
PR-27614 (C_At_Rv)	cgtgcgauttagcaggcgggagcagc
PR-27773 (C_AtSMT2_Fw)	acttttgcagtacuaaccgcaggactctctgaccctg
PR-27774 (C_AtSMT2_Rv)	cacgcgauttaagaagattcctcgggag

Yeast transformation

The yeast vectors were integrated into different previously characterized intergenic loci in the *Y. lipolytica* genome as described in Holkenbrink *et al*, 2018;see also Table 18 below. Integration vectors were digested with *Not*I enzyme (New England BioLabs) at 37°C for 1 hr and the digested product purified from solution using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The purified DNA was transformed using the lithium acetate transformation protocol as described by Holkenbrink *et al*, 2018. Correct integration was verified by colony PCR using Taq DNA Polymerase Master Mix RED (Ampliqon) with vector-specific primers and primers complementary to the genomic region adjacent to the integration site.

For marker-mediated gene deletion, *Y. lipolytica* strains were transformed with BioBricks assembled by USER reaction as detailed above. Transformants were selected on antibiotic supplemented plates and correct transformants confirmed by colony PCR. Marker removal was performed by transformation of the strains with a Cre-recombinase episomal vector. Marker removal was confirmed by colony PCR.

Yeast cultivation

Yeast strains were inoculated into 2.5 ml YPD in 24-deepwell plates with air-penetrable lids (EnzyScreen, Netherlands). The plates were incubated at 30°C with 300 rpm agitation at 5 cm orbit cast for 24 hours. The cultures were then diluted to OD600 0.1 in 2.5 ml fresh YPD-media with 80 g/l glucose and grown for a further 72 hours at 30°C with 300 rpm agitation. All cultivations were performed in triplicate. Dry cell weight (DCW) was measured at the end of cultivation: 1 ml of culture broth was transferred into a pre-weighed 2 ml microcentrifuge tube, centrifuged (3000 g, 5 min) and the supernatant was discarded. The cells were washed twice with deionized water (1 ml). The cell pellet was dried at 60°C for 7 days before the final weight was measured.

Sterol analysis

For sterol extraction, 1 ml of culture broth was transferred into a 2 ml microcentrifuge tube, centrifuged and the supernatant was discarded. The cells were washed twice with deionized water (1 ml). The cell pellet was resuspended in 10% w/v methanolic potassium hydroxide (500 µl) and transferred to a 1 ml glass vial for saponification. The suspension was incubated at 70°C for 2 hours with vortexing at 15 minute intervals. The saponified samples were then vortexed and spiked with 50

µl of internal standard (1 mg/ml epicoprostanol in absolute ethanol). 500 µl of n-hexane was added to each sample for extraction of the free sterol component. Samples were vortexed and the organic phase transferred to a 2 ml microcentrifuge tube. The extraction step was repeated in a further 500 µl of n-hexane. The combined hexane phases were left overnight at room temperature for evaporation of the solvent. The resulting crystals were resuspended in 50 µl of n-hexane for concentration at the bottom of the tube and left at room temperature overnight for final drying. Samples were then stored at 4°C prior to analysis.

Sterols were derivatized with 20 µl Tri-Sil (Sigma) and then briefly vortexed before direct injection into an Agilent Technologies (Palo Alto, CA, USA) 7890A gas chromatograph connected to an Agilent Technologies 5975C MSD mass spectrometer (for gas chromatography–mass spectrometry (GC-MS)) and eluted over an Agilent DB5 column using a splitless injection at 250°C with a standard GC program at 170 °C for 1 min, ramped to 280°C at 20°C min^{-1} and monitoring between 50 and 550 amu.

Sterols were identified by comparison of their retention time relative to cholesterol and mass spectra data available from the NIST (National Institute of Standards and Technology) mass spectral library and (Zu et al. 2021). Sterols were quantified by calculating the ratio of the peak area of the targeted sterol to that of the internal standard. The mass of each sterol in the sample was obtained by multiplying the ratio with the mass of the internal standard. Compound identification (using target ions) and quantification were carried out with ChemStation Enhanced Data Analysis (v.E.01.00).

Results

Construction of a *Yarrowia lipolytica* strain capable of synthesising a sterol surrogate

The gene encoding squalene-tetrahymanol cyclase from *Tetrahymena thermophilia* (*TtSTC*) was integrated into the ST9100 platform strain genome under the control of the PrGPAT promoter, previously characterised as a weak promoter (Holkenbrink *et al*, 2018). This promoter was selected to drive minimal expression of *TtSTC* to limit diversion of carbon flux away from the main sterol pathway. As noted above, the promoter described as 'PrGPAT' in Holkenbrink *et al*, 2018 (sequence provided in the supplementary information of that paper) and used in this study does not belong to the *Y. lipolytica* GPAT gene (YALI1_C00230g, Y. lipolytica W29/CLIB89 genome assembly, Magnan *et al*, 2016), but is instead the promoter of a putative gene (YALI1_C00209g). The resulting strain ST11005 produced 0.4 mg/g dry cell weight tetrahymanol, comprising 14.0% of the total sterol fraction (Fig. 11).

Construction of *Yarrowia lipolytica* strains incapable of synthesising ergosterol

As noted above, the tetrahymanol producing strain ST11005 was used for the construction of strains incapable of synthesising ergosterol. Firstly, to create a ΔERG5 strain, the ERG5 gene (sterol C-22 desaturase, YALI1_A18344g) was deleted by marker-mediated knock-out. A knock-out cassette was constructed comprising a hygromycin resistance marker (HphMX) flanked by 1 kb homology arms corresponding to 1 kb upstream and downstream of the ERG5 coding sequence. This construct was used to transform ST11005, generating ST11014. The HphMX resistance marker was subsequently looped out by Cre-Lox recombination. The resulting strain ST11027 produced 3.1 mg/g DCW ergosta-

5,7-dienol, comprising 60.0% of the total sterol fraction. The strain also produced 5.2 mg/g DCW tetrahymanol, comprising 36.7% of the total sterol fraction.

To create a ΔERG4ΔERG5 strain, the ERG4 gene (delta-24 sterol reductase, YALI1_D24361g)) was deleted by HphMX marker-mediated knockout in an analogous manner. The resulting strain ST11040 produced 3.1 mg/g DCW ergosta-5,7,24(28)-trienol, comprising 20.0% of the total sterol fraction. The strain also produced 15.8 mg/g DCW tetrahymanol, comprising 75.2% of the total sterol fraction.

To create a ΔERG5ΔERG6 strain, the gene encoding ERG6 (sterol methyl transferase, YALI1_F12138g) was deleted from ST11027 by HphMX marker-mediated knockout in an analogous manner, generating strain ST11325. The HphMX resistance marker was subsequently looped out by Cre-Lox ecombination. The resulting strain ST11330 produced 0.2 mg/g DCW zymosterol, comprising 0.6% of the total sterol fraction. The strain also produced 36.0 mg/g DCW tetrahymanol, comprising 99.4% of the total sterol fraction.

Construction of campesterol-producing strains

The ERG5 knock-out strain ST11027 was used for the construction of campesterol-producing strains. Delta-7 sterol reductase gene variants were expressed under the control of the PrTEFintron promoter, generating strains ST11066 to ST11075. The highest campesterol yield, 40.2 mg/g DCW, was obtained in the strain ST11071 expressing the *Coccomyxa subellipsoidea* delta-7 sterol reductase (Fig. 12).

Optimisation of campesterol-producing strains

Campesterol can be produced by expressing a delta-7 sterol reductase in a base strain, a strain with down-regulated ERG5 expression, or a strain in which ERG5 is deleted. *Ectocarpus siliculosus* delta-7 sterol reductase was expressed under the control of the PrTEFintron promoter in strain ST9100 and strain ST11027 (ΔERG5), generating strains ST10924 and ST11069. The ERG5 promoter was truncated to 48bp in strain ST10924, generating strain ST91034 (ERG5_trPr_48bp). The *PAH1* gene (YALI1_D35593g) was also knocked out in ST10924 and ST11069 to increase the amount of membranes and capacity for sterol accumulation. Gene deletion was achieved by CRISPR-Cas9 and a 1kb repair template comprising homology arms corresponding to 500bp up- and downstream of the PAH1 coding sequence. ERG5 deletion results in the greatest increase in campesterol yield. The highest campesterol yield, 41.5 mg/g DCW, was obtained in the strain ST11197 (Fig. 13).

Construction of 24-methlyenecholesterol-producing strains

The ΔERG5ΔERG4 strain ST11040 was used for the construction of 24-methlyenecholesterol-producing strains. Delta-7 sterol reductase gene variants were similarly expressed under the control of the PrTEFintron promoter, generating strains ST11056 to ST11065. All ten variants were tested as the likely substrate of delta-7 reductase is ergosta-5,7,24(28)-trienol in these strains, compared to ergosta-5,7-dienol in the campesterol-producing strains. [Nb. predicted substrates of delta-7 sterol reductase were inferred from parental strains and from the biochemical pathway. As noted above, ergosta-5,7-dienol accumulates in Δerg5 strain ST11027 (from which campesterol strains are derived by addition of delta-7 sterol reductase)]. Ergosta-5,7,24(28)-trienol accumulates in ΔERG4-ERG5

strain ST11040 (from which 24-methylenecholesterol strains are derived by addition of delta-7 sterol reductase)]. Thus, the substrate specificity and catalytic activity of the delta-7 sterol reductase variants may differ between backgrounds.

The highest 24-methylenecholesterol yield, 47.7 mg/g DCW, was obtained in the strain ST11064 expressing the *Tetraselmis* sp. GSL018 delta-7 reductase under the control of the PrTEFintron promoter. (Fig. 14).

Discussion

The total sterol content is greater in all strains incapable of producing ergosterol than those capable of producing ergosterol. It is interesting to note that the ergosterol contents in the platform strain ST9100 and the strain ST11005 expressing *TtSTC* are not greater than that of the parent strain ST6512 which does not contain modifications for increased terpenoid production. It is therefore anticipated that the ergosterol pathway is tightly regulated to control ergosterol concentration in the cell. This may partly be achieved by end-product feedback inhibition. This mechanism would be expected to be highly specific to ergosterol, to prevent interference by structurally similar early intermediates of the ergosterol pathway. Hence, sterol accumulation is greater in strains lacking ergosterol, particularly in those producing non-native sterols. These sterols are likely to be esterified and stored in lipid droplets to prevent toxicity.

Not all strains capable of producing campesterol require a tetrahymanol substitute. Campesterol can be produced in strains expressing a heterologous delta-7 sterol reductase alone, or strains expressing a heterologous delta-7 sterol reductase with partial reduction of *ERG5* expression. However, a strain capable of producing a small amount of tetrahymanol seems to accumulate more campesterol than a strain that produces a small amount of ergosterol. It is possible that this is because tetrahymanol substitutes for ergosterol function in the membrane but does not down-regulate the pathway by end-product inhibition in the same way that ergosterol might. Increasing the amount of cellular membranes in a strain producing campesterol and tetrahymanol has a greater effect on the accumulation of tetrahymanol than campesterol. This suggests that tetrahymanol accumulates in the membrane. Campesterol is likely esterified and stored in lipid droplets.

Similarly, the tetrahymanol content is greater in all strains incapable of producing ergosterol than those capable of producing ergosterol. Whilst this is expected if overall flux through the ergosterol and preceding pathways is increased, the percentage of tetrahymanol relative to the total sterol component is also increased. This may suggest that whilst ergosterol is the preferred sterol, tetrahymanol may be used in the absence of a suitable alternative.

As such, ERG4 has been previously identified as an essential gene in *Y. lipolytica* wild-type strain CLIB89 (W29, ATCC® 20460™) by transposon mutagenesis (Patterson et al. 2018). The main Erg4p substrate, ergosta-5,7,22,24(28)-tetraenol, may be unable to support normal cellular function alone, for example due to toxic effects on membrane structure and properties. However, in strains capable of synthesising tetrahymanol, ERG4 is non-essential. In ST11040, ergosta-5,7,24(28)-trienol accumulates, yet tetrahymanol comprises 75.2% of the total sterol fraction. This suggests that tetrahymanol can better substitute for ergosterol function in the cell. Similarly, ERG6 has been

classified as an essential gene in *Y. lipolytica* by transposon mutagenesis (Patterson et al. 2018) and CRISPR-Cas9 mediated gene disruption (Schwartz et al, 2019). However, in strains capable of synthesising tetrahymanol, *ERG6* is non-essential. In ST11330, zymosterol accumulates, yet tetrahymanol comprises 99.4% of the total sterol fraction. This again suggests that tetrahymanol can better substitute for ergosterol function in the cell.

Engineered *Y. lipolytica* strains therefore hold great potential for the synthesis of non-native sterol structures, due to the capacity of this oleaginous yeast to store and accumulate sterols. It is expected that sterol content could be improved by further strain modification and scale-up of cultivations.

Construction of a β-sitosterol-producing strain

The campesterol-producing strain ST11071 was used for the construction of a β-sitosterol-producing strain (Fig. 17A). The C-28 sterol methyltransferase gene variant from *C. quinoa* was expressed under the control of the PrTEFintron promoter. The resulting strain ST11804 produced 1.2 mg/g DCW β-sitosterol. To further increase β-sitosterol production, two additional C-28 sterol methyltransferase gene variants from *A. thaliana* and *A. trichopoda* were expressed under the control of the PrGPD and PrTEFintron promoters, respectively. The resulting strain ST12139 produced 5.4 mg/g DCW β-sitosterol.

Construction of an isofucosterol-producing strain

The 24-methylenecholesterol-producing strain ST11064 was used for the construction of an isofucosterol-producing strain (Fig 17A). The C-28 sterol methyltransferase gene variant from *C. quinoa* was expressed under the control of the PrTEFintron promoter. The resulting strain ST11803 produced 5.0 mg/g DCW isofucosterol. To further increase isofucosterol production, two additional C-28 sterol methyltransferase gene variants from *A. thaliana* and *A. trichopoda* were expressed under the control of the PrGPD and PrTEFintron promoters, respectively. The resulting strain ST12108 produced 20.2 mg/g DCW isofucosterol.

Construction of a desmosterol-producing strain

The ΔERG5ΔERG6 strain ST11330 was used for the construction of a desmosterol-producing strain (Fig 17B). The delta-7 sterol reductase gene variant from *L. drancourtii* was expressed under the control of the PrTEFintron promoter, generating strain ST11346. Strain ST11346 produced 9.4 mg/g DCW desmosterol. Note, as explained in the discussion below, the deletion of ERG5 is not deemed essential.

Construction of a cholesterol-producing strain

The desmosterol-producing strain ST11346 was used for the construction of a cholesterol-producing strain (Fig 17B). The delta-24 sterol reductase gene variants from *D. rerio* and *M. musculus* were expressed under the control of the PrTEFintron promoter, generating strains ST11829 and ST11830, respectively. Strain ST11829 produced 26.6 mg/g DCW cholesterol and strain ST11830 produced 13.9 mg/g DCW cholesterol.

Discussion

Again, since tetrahymanol is able to substitute for ergosterol function in the cell, the ergosterol biosynthesis pathway could subsequently be disrupted without compromising cell viability. The native C-22 sterol desaturase gene ERG5 was deleted together with either ERG4 or ERG6 to prevent ergosterol synthesis and allow diversion of flux toward the non-native sterols upon the introduction of additional heterologous genes. The single gene deletion of ERG6 in combination with the expression of a heterologous delta-7 sterol reductase is sufficient for desmosterol production. In the case of strain ST11346, the deletion of ERG5 is not strictly necessary to obtain desmosterol but was included to facilitate the subsequent construction of cholesterol-producing strains.

In some strains, additional intermediate sterols may accumulate together with the main desired sterol. For example, the *D. rerio* delta-24 sterol reductase gene variant in ST11829 is more efficient at reducing desmosterol to cholesterol than the *M. musculus* variant in ST11830. Hence, ST11829 contains 0.3 mg/g DCW desmosterol and 26.6 mg/g DCW cholesterol, compared to 4.7 mg/g DCW desmosterol and 13.9 mg/g DCW cholesterol in strain ST11830.

The *C. quinoa* C-28 sterol methyltransferase gene variant appeared to have weak activity and using this methyltransferase the production of 24-ethyl sterols was comparatively low: strain ST11804 produced 1.2 mg/g DCW β-sitosterol and 3.0 mg/g DCW of the precursor campesterol. Strain ST11803 produced 5.0 mg/g DCW isofucosterol and only 10.2 mg/g DCW of the precursor 24-methylenecholesterol. Consequently, two additional sterol methyltransferase gene variants were introduced into these strains to increase the relative content of the 24-ethyl sterols. The difference in the relative content of 24-ethyl sterols in the resulting strains ST12139 and ST12108 may be due to a difference in substrate specificity of the sterol methyltransferase enzymes toward the respective 24-methyl sterol precursors in each strain.

As previously noted above, it is anticipated that the ergosterol pathway is tightly regulated to control ergosterol concentration in the cell. This may partly be achieved by end-product feedback inhibition. This mechanism would be expected to be highly specific to ergosterol, to prevent interference by structurally similar early intermediates of the ergosterol pathway. Hence, sterol accumulation is greater in strains lacking ergosterol, particularly in those producing non-native sterols. These sterols are likely to be esterified and stored in lipid droplets to prevent toxicity. The amount of tetrahymanol remained less than 20 mg/g DCW in the strains producing each of the non-native sterols.

The examples given demonstrate that *Y. lipolytica* has the capacity to accumulate and store a range of different sterol structures. Engineered *Y. lipolytica* strains therefore hold great potential for the synthesis of many non-native sterol structures, and the dominant sterol may be tailored to suit different applications. It is expected that sterol content could be improved by further strain modification and scale-up of cultivations.

Additional reference:

Zu P, Koch H, Schwery O, Pironon S, Phillips C, Ondo I, Farrell IW, Nes WD, Moore E, Wright GA, Farman DI & Stevenson PC (2021) Pollen sterols are associated with phylogeny and environment but not with pollinator guilds. *New Phytol.* **230:** 1169–1184

Example 4 - Engineering of *Yarrowia lipolytica* for production of a mixture of non-native sterols

Materials and Methods

Strains, culture conditions and chemicals

Escherichia coli strain DH5α was used for plasmid construction as above.

The strain ST9100 was again used to construct all non-native sterol-producing strains. All strains are detailed in Tables 5a and 5b.

Y. lipolytica was grown at 30°C on yeast extract peptone dextrose (YPD) media containing 10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose, supplemented with 20 g/l agar for preparation of solid media. For selection, either nourseothricin (250 mg/l) or hygromycin (400 mg/l) was added to the media.

Cultivation of strains for sterol production was again performed in yeast extract peptone medium containing 80 g/l glucose.

Chemicals were obtained, if not indicated otherwise, from Sigma-Aldrich. Nourseothricin was purchased from Jena BioScience GmbH (Germany).

Plasmid construction

The coding sequences for delta-7 sterol reductase from Tetraselmis sp. GSL018 (GenBank accession: JAC78771.1), squalene-tetrahymanol cyclase from *Tetrahymena thermophilia* (accession: XP_001026696.2), delta-24(25) sterol reductase from *Solanum lycopersicum* (accession: BAQ55273.1), C-28 sterol methyltransferase from *Chenopodium quinoa* (accession: XP_021737090.1) and delta-24(28) sterol reductase from *Solanum tuberosum* (accession; AB839749.1) were codon-optimized for *Y. lipolytica* and synthesised as GeneArt String DNA fragments by ThermoFisher Scientific. The codon-optimized sequences are listed in Tables 4a and b.

The plasmids, biobricks, and primers used in this study are listed in Tables 15-17, respectively. BioBricks were again amplified by PCR. The PCR mix contained 32 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 1 µl 10mM dNTP, 1.5 µl $MgCl_2$, 2.5 µL 10 µM Fw primer, 2.5 µl 10 µM Rv primer, 0.2 µl Phusion® U polymerase, and 10 ng DNA template. Reactions were multiplexed 8x per BioBrick. The cycling program was: 98°C for 1 min, 30 cycles of [98°C for 10 sec, gradient from 50 to 58°C for 20 sec, 72°C for 30 s/kb], 72°C for 5 min, pause at 10°C. The gene fragments were purified from agarose gels using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Biobricks were assembled into the EasyCloneYALI vectors with USER cloning (Holkenbrink et al. 2018). The USER reactions were transformed into *E. coli* and correct assemblies were verified by Sanger sequencing (Eurofins).

Table 15: Plasmids for engineering of *Yarrowia lipolytica* for production of a mixture of non-native sterols

Plasmid name	Parent plasmid/ Reference	BioBricks
pCfB5935 (pIntA-1-HphMx-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6633 (pNat-YLgRNA2_IntE_1)	(Holkenbrink *et al*, 2018)	
pCfB6637 (pNat-YLgRNA3_IntE_3)	(Holkenbrink *et al*, 2018)	
pCfB6638 (pNat-YLgRNA2_IntE_4)	(Holkenbrink *et al*, 2018)	
pCfB6677 (pIntE_1-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6681 (pIntE_3-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB4783 (pIntE_3-Nat-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pCfB6679 (pIntE_4-TPex20-TLip2)	(Holkenbrink *et al*, 2018)	
pBP8009 (pIntF_3-TPex20-TLip2)	(Sáez-Sáez *et al*, 2020)	
pCfB10783(gRNA_IntE_5)	Dr. J. Dahlin, DTU CfB, Denmark	
pBP8003 (pNat-YLgRNA4-IntF_3)	(Sáez-Sáez *et al*, 2020)	
pCfB8861 (pHyg-YLgRNA3_IntE_4)	(Arnesen *et al*, 2020)	
pBP8660 (pIntE_5-TPex20-TLip2)	Dr. K.R. Kildegaard, Biophero ApS, Denmark	
pCfB6611 (pNat-PrExp-Cre)	pCfB4158 (pPrExp-Cre) - (Holkenbrink *et al*, 2018)	
pCfB6612 (pHph-PrExp-Cre)	pCfB6611 (backbone amplified with PR-21844 and PR-10593)	BB2816 (PrExp-HphMx-Ttef)
pCfB10257 (IntE4_PrTEF->DHCR7_Tsp)	pCfB6679	BB3879 (Tefint->):BB4884 (PrTEF_DHCR7Ts)
pCfB10494 (IntE3_PrGPAT->TtSTC)	pCfB6681	BB1617 (PrGPAT): BB5100 (TtSTC_USER)
pCfB10841 (IntF3_PrDGA1->ERG4)	pBP8009	BB1616 (PrDGA1): BB5362 (ERG4_USER)
pCfB10842 (IntF3_PrGPAT->ERG4)	pBP8009	BB1617 (PrGPAT): BB5362 (ERG4_USER)
pCfB10851 (IntE1_PrGPAT->SMT2_Cquinoa)	pCfB6677	BB1617 (PrGPAT->): BB5371 (PrGPAT_SMT2Cq)
pCfB10853 (IntE5_PrGPAT->DHCR24_Slycopersic)	pBP8660	BB1617 (PrGPAT->): BB5373 (PrGPAT_DHCR24Sl)
pCfB10919 (Int_3 NatMX_PrGPAT->SMT2_Cquinoa)	pCfB4783	BB5371 (PrGPAT_SMT2Cq)
pCfB10948 (IntF3_PrDGA1->StSSR1)	pCfB8009	BB1616 (PrDGA1): BB5456 (StSSR1_USER)

Full reference for Sáez-Sáez et al. (2020):Sáez-Sáez J, Wang G, Marella ER, Sudarsan S, Cernuda Pastor M & Borodina I (2020) Engineering the oleaginous yeast Yarrowia lipolytica for high-level resveratrol production. *Metab. Eng.* **62:** 5

Table 16: Biobricks for engineering of *Yarrowia lipolytica* for production of a mixture of non-native sterols

Biobrick name		Template/Reference	Forward primer	Reverse primer
BB1616 (PrDGA1->)		(Holkenbrink *et al*, 2018)		
BB1617 (PrGPAT->)		(Holkenbrink *et al*, 2018)		
BB3879 (PrTefint->)		(Arnesen *et al*, 2020)		
BB4884 (PrTEF_DHCR7Ts)		*Tetraselmis* sp. GSL018 delta-7 sterol reductase codon optimised	PR-27581	PR-27582
BB2816 (PExp-HphMx-Ttef)		pCfB5935 - (Holkenbrink et al, 2018)	PR-15604	PR-22297
BB5041 (LoxP_HphMX)		pCfB5935 - (Holkenbrink et al, 2018)	PR-27972	PR-27973
BB5101 (LoxP_NatMX)		pCfB4788	PR-27972	PR-27973
BB5091 (*erg4*_1kbUP_U1)		ST9100 gDNA	PR-27891	PR-28358
BB5092 (*erg4*_1kbDOWN_U2)		ST9100 gDNA	PR-27893	PR-27894
BB5093 (*erg5*_1kbUP_U1)		ST9100 gDNA	PR-27895	PR-28359
BB5094 (*erg5*_1kbDOWN_U2)		ST9100 gDNA	PR-27897	PR-27898
BB5097 (*erg4*_HphMX_KO)		BB5091 + BB5092 + BB5041 USER reaction	PR-27891	PR-27894
BB5098 (*erg5*_HphMX_KO)		BB5093 + BB5094 + BB5041 USER reaction	PR-27895	PR-27898
BB5100 (TtSTC_USER)		*Tetrahymena thermophilia* squalene-tetrahymanol cyclase codon optimised	PR-28361	PR-28362
BB1135 (Easy Clone vector backbone)		pCfB6677	PR-11110	PR-11111
BB5362 (ERG4_USER)		ST9100 gDNA	PR-29139	PR-29140
BB5371 (PrGPAT_SMT2Cq)		*Chenopodium quinoa* sterol methyltransferase codon optimised	PR-27621	PR-27786
BB5373 (PrGPAT_DHCR24Sl)		*Solanum lycopersicum* delta-24 sterol reductase codon optimised	PR-27599	PR-27764
BB5456 (StSSR1_USER)		*Solanum tuberosum* delta-24(28) sterol reductase codon optimised	PR-29404	PR-29405

Table 17: Primers for engineering of *Yarrowia lipolytica* for production of a mixture of non-native sterols

Primer name	Sequence (5' → 3')
PR-14617 (vector verification *E. coli* cPCR)	tatccctgtgttgaatc
PR-14619 (vector verification *E. coli* cPCR)	tatcgacccagttagc

PR-8859 (integration verification YI cPCR)	aagtgtggatggggaagtgag
PR-14442 (IntE_1 verification YI cPCR)	agttgtgaccaagacaaatg
PR-14398 (IntE_1 verification YI cPCR)	cacgcgaUgttagaagcaattggagaag
PR-14576 (IntE_3 verification YI cPCR)	cacgcgautgaaggaaatgcctaaaacc
PR-14835 (IntE_3 verification YI cPCR)	cacgcacgccattctataag
PR-14592 (IntE_4 verification YI cPCR)	acgcgauttaacactggaccgtactgc
PR-20880 (IntE_4 verification YI cPCR)	attgctaagcgaccatagac
PR-14837 (IntF_3 verification YI cPCR)	acatgctcgcgcctcgatag
PR-14584 (IntF_3 verificiationYI cPCR)	cacgcgautttggtcgtcgcccaacaag
PR-26834 (IntE_5 verification YI cPCR)	gggacaagtgcaatcgttcacttg
PR-26835 (IntE_5 verification YI cPCR)	cgttgctgttgccacaatggac
PR-27581 (A_Ts_Fw)	actttttgcagtacuaaccgcagaagcgagcctccaagaccc
PR-27582 (A_Ts_Rv)	cacgcgauttagaagatgtagggcacgatc
PR-27891 (Erg4Rep_P1F)	ctctcaacaccttcaccgc
PR-28358 (Erg4Rep_P1R_U1)	atcgcacgugataagcttagtgagcgaatgg
PR-27893 (Erg4Rep_P2F)	gtgcaggutgtgtggttgcgaaggaag
PR-27894 (Erg4Rep_P2R)	gcactcaaaataccccgttc
PR-27895 (Erg5Rep_P1F)	ctcggtttgttgcagcagg
PR-28359 (Erg5Rep_P1R_U1)	atcgcacgutggtccgtatcgtgaaatgg
PR-27897 (Erg5Rep_P2F)	gtgcaggugggcggagttgtgttgtg
PR-27898 (Erg5Rep_P2R)	ggtcggctatccaatacatctc
PR-27972 (HphMX_F_U1)	cgtgcgautcagctgaagcttcgtac
PR-27973 (HphMX_R_U2)	acctgcacugcataggccactagtgg
PR-15604 (PrExp_for fusion->_U2_fw)	agctactgauaaggagtttggcgcccgtt
PR-22297 (Ttef_U_EpiVecYL_rv)	aagcctgcugaattcggacacgggcat
PR-21844 (gRNA_opened_rv)	atcagtagcuctactgtatattcaagcaag
PR-10593 (Fragment2EpiVecYL_fw)	agcaggctuggaggcgacgtggcag
PR-28361 (TtSTC_F_U3)	atctgtcaugccacaatgaagaagatcctcatcggtc
PR-28362 (TtSTC_Rv)	cacgcgauttagatgttctgcttctggacg
PR-22830 (Derg5_chk)	tcatactcaccgaaacgtg
PR-27961 (Derg5_chk)	gttccaatgcctggcaag
PR-27634 (Derg5_chk)	acttctctctctcacaccacc
PR-27635 (Derg5_chk)	ctgagggctctgttggtgaag
PR-27636 (Derg5_chk)	accagtgtggttgtaaggatg
PR-11138 (Derg4_chk)	agcaatggguaaaaagcctgaactcaccgc
PR-27631 (Derg4_chk)	cctgatattggtgatcctcc
PR-27632 (Derg4_chk)	agagccttgtttccgaggtg
PR-27633 (Derg4_chk)	atacaatcccataggctggc
PR-28001 (Derg4_chk)	cgtgcgaugcttgccctggactacatcttg
PR-27599 (B_Sl_Fw)	atctgtcaugccacaatgtctgacgccaaggctc

PR-27764 (B_SI_Rv)	cacgcgauttagttggcctcagggg
PR-27621 (C_Cq_Fw)	atctgtcaugccacaatggactctatggccctg
PR-27786 (C_Cq_Rv)	cacgcgauttaagagtcagacttggc
PR-29139 (ERG4_ORF_F_U3)	atctgtcaugccacaatgtctgctgttcgacaacg
PR-29140 (ERG4_ORF_Rv)	cacgcgauttagaaaacataagggatgaagatcc
PR-28981 (Backbone_amp_Tlip_F)	atcgcgtgcautccttctgttcggaatcaacct
PR-29404 (StSSR1_F_U3)	atctgtcaugccacaatgaccgacgtccagg
PR-29405 (StSSR1_Rv)	cacgcgauttagtcctcgggctcgtc

Yeast transformation

Again, the yeast vectors were integrated into different previously characterized intergenic loci in the *Y. lipolytica* genome as described in Holkenbrink *et al*, 2018; see also Table 18 below. Integration vectors were digested with *Not*I enzyme (New England BioLabs) at 37°C for 1 hr and the digested product purified from solution using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The purified DNA was transformed using the lithium acetate transformation protocol as described by Holkenbrink *et al*, 2018. Correct integration was verified by colony PCR using Taq DNA Polymerase Master Mix RED (Ampliqon) with vector-specific primers and primers complementary to the genomic region adjacent to the integration site.

For marker-mediated gene deletion, *Y. lipolytica* strains were transformed with biobricks assembled by USER reaction as detailed in Table 16. Transformants were selected on antibiotic supplemented plates and correct transformants confirmed by colony PCR. Marker removal was performed by transformation of the strains with a Cre-recombinase episomal vector. Marker removal was confirmed by colony PCR.

Yeast cultivation

Yeast strains were inoculated into 2.5 ml YPD in 24-deepwell plates with air-penetrable lids (EnzyScreen, Netherlands). The plates were incubated at 30°C with 300 rpm agitation at 5 cm orbit cast for 24 hours. The cultures were then diluted to OD600 0.1 in 2.5 ml fresh YPD-medium with 80 g/l glucose and grown for a further 72 hours at 30°C with 300 rpm agitation. All cultivations were performed in triplicate. Dry cell weight was measured at the end of cultivation: 1 ml of culture broth was transferred into a pre-weighed 2 ml microcentrifuge tube, centrifuged (3000 g, 5 min) and the supernatant was discarded. The cells were washed twice with deionized water (1 ml). The cell pellet was dried at 60°C for 7 days before the final weight was measured.

Sterol analysis

For sterol extraction, 1 ml of culture broth was transferred into a 2 ml microcentrifuge tube, centrifuged and the supernatant was discarded. The cells were washed twice with deionized water (1 ml). The cell pellet was resuspended in 10% w/v methanolic potassium hydroxide (500 µl) and transferred to a 1 ml glass vial for saponification. The suspension was incubated at 70°C for 2 hours with vortexing at 15 minute intervals. The saponified samples were then vortexed and spiked with 50 µL of internal standard (1 mg/ml epicoprostanol in absolute ethanol). 500 µl of n-hexane was added to each sample for extraction of the free sterol component. Samples were vortexed and the organic

phase transferred to a 2 ml microcentrifuge tube. The extraction step was repeated in a further 500 µl of n-hexane. The combined hexane phases were left overnight at room temperature for evaporation of the solvent. The resulting crystals were resuspended in 50 µl of n-hexane for concentration at the bottom of the tube and left at room temperature overnight for final drying. Samples were then stored at 4°C prior to analysis.

Sterols were derivatized with 20 µl Tri-Sil (Sigma) and then briefly vortexed before direct injection into an Agilent Technologies (Palo Alto, CA, USA) 7890A gas chromatograph connected to an Agilent Technologies 5975C MSD mass spectrometer (for gas chromatography–mass spectrometry (GC-MS)) and eluted over an Agilent DB5 column using a splitless injection at 250°C with a standard GC program at 170°C for 1 min, ramped to 280°C at 20°C min^{-1} and monitoring between 50 and 550 amu.

Sterols were identified by comparison of their retention time relative to cholesterol and mass spectra data available from the NIST (National Institute of Standards and Technology) mass spectral library and Zu et al. 2021. Sterols were quantified by calculating the ratio of the peak area of the targeted sterol to that of the internal standard. The mass of each sterol in the sample was obtained by multiplying the ratio with the mass of the internal standard. Compound identification (using target ions) and quantification were carried out with ChemStation Enhanced Data Analysis (v.E.01.00).

Bioreactor fed-batch cultivation

The Ambr® 250 system (Sartorius Stedim Biotech, Göttingen, Germany) was used to carry out 250 ml fed-batch fermentation in duplicate. Strain ST11064 was re-streaked from glycerol stocks onto a YPD agar plate and incubated at 30°C for 48 h. The preculture was prepared by inoculating strain ST11064 biomass from the plate into 50 ml YPD medium in a 250 ml shake flask and incubating at 30°C for 24 hours with 250 rpm agitation. Five ml of preculture was used to inoculate 115 ml batch medium, to a starting OD_{600} of 0.25. For Ambr® 250 cultivation, the batch medium comprised mineral medium supplanted with yeast extract (10 g/l) and citric acid (20 g/l). The mineral medium was prepared with 0.5 g/l $MgSO_4 \cdot 7H_2O$, 14.4 g/l KH_2PO_4, 0.1% (v/v) vitamin solution, 0.2% (v/v) trace metal solution as described in (Jensen *et al*, 2014), but with 3.4 g/l NH_4Cl and glycerol as the carbon source (40 g/l)

Temperature was held constant at 30°C. Dissolved oxygen was maintained above 20% by using a cascade of stirring speed ranging from 600 to 3000 rpm and aeration up to 1 vvm. The pH was maintained at 5.5 by the automatic addition of 1M NaOH and 2.6M H_3PO_4 phosphoric acid. Antifoam 204 (Sigma) was added automatically. The feed medium comprised 250 g/l glycerol. Feeding was automatically initiated once CER dropped below 50% at the end of the batch phase. Feeding was set to a constant rate of 0.9 ml/h. Samples were taken every 6 h for the first 24 hours, and then every 12 hours, and immediately frozen until preparation for analysis. CDW and sterol content were determined from 1 ml samples as described above for small scale cultivation.

For larger scale fermentation, strains were cultivated by fed-batch fermentation in a 5 L bioreactor (BIOSTAT® B-DCU, Sartorius, Goettingen, Germany). All fermentations were carried out in duplicate. For each of strains ST4842, ST11005 and ST12178, the strain was re-streaked from glycerol stocks onto a YPD agar plate and incubated at 30°C for 24 h. The preculture was prepared by inoculating strain biomass from the plate into 50 ml YPD medium in a 250 ml shake flask and incubating at 30°C

for 24 hours with 250 rpm agitation. The volume of pre-culture required to inoculate 2L batch medium to a starting OD_{600} of 2.5 was centrifuged for 10 min at 4000xg and concentrated in 10 ml volume. This cell suspension was used to inoculate the bioreactors. The bioreactors were equipped with pH, pO2 and temperature probes. Temperature was held constant at 30°C. Dissolved oxygen was maintained above 20% by adjusting stirring between 600 and 1200 rpm and aeration (via a horseshoe sparger) between 0.5 and 3 standard-litre per min (SPLM). The pH was kept at 5.5 by automatic addition of 5 M NaOH. Antifoam A (Sigma) was added as required.

The batch media comprised of mineral medium supplanted with yeast extract (20 g/l) and peptone (40 g/l). The mineral medium was prepared with 0.5 g/l $MgSO_4 \cdot 7H_2O$, 14.4 g/l KH_2PO_4, 0.1% (v/v) vitamin solution, 0.2% (v/v) trace metal solution as described in (Jensen *et al*, 2014), 40 g/l glucose and 1 ml/l antifoam A (Sigma). The feed contained 5 g/l $MgSO_4 \cdot 7H_2O$, 30 g/l KH_2PO_4, 1% (v/v) vitamin solution, 2% (v/v) trace metal solution as described in (Sáez-Sáez *et al*, 2020), with 300 g/l glucose. An exponential feeding profile was programmed, and feeding was initiated 24 h after inoculation. Feed rate, F (ml/h) followed the profile F=10*e^(0.05*t), where t = time (h) from the start of feeding. After 36 h of exponential feeding, the feed was switch to a constant rate of 75 ml/h until the end of fermentation.

Samples were taken every 8 hours for the first 24 hours, and then every 12 hours, to measure CDW, sterol content, OD600 and glucose concentration. CDW and sterol content were determined from 1 ml samples as described above for small scale cultivation. OD600 was measured with a VWR NanoPhotometer 7122. During fermentation, 1 ml culture broth was centrifuged and the supernatant was used to measure glucose concentration via a glucose HK assay kit (Sigma). The supernatant was then filtered and frozen until further analysis. Glucose was later quantified on a Dionex Ultimate 3000 HPLC system equipped with a RI-101 Refractive Index Detector (Dionex) refractive index detector. An Aminex HPX-87H column 7.8 mm × 300 mm (Bio-Rad) with a Micro-Guard Cation H+ guard column 4.6 mm × 30 mm heated to 30°C was injected with 10 µl sample. The mobile phase consisted of 5 mM H_2SO_4 with an isocratic flow rate of 0.6 ml/min, which was held for 15 min. HPLC data were processed using Chromeleon 7.2.9 software (Thermo Fisher Scientific). Glucose was identified and quantified using authentic standards. Glucose concentrations were calculated from peak area by extrapolation from a six-point calibration curve regression.

Results

Construction of a Yarrowia lipolytica strain capable of synthesising a sterol surrogate

The gene encoding squalene-tetrahymanol cyclase from *Tetrahymena thermophilia* (*TtSTC*) was integrated into the ST9100 platform strain genome under the control of the *PrGPAT* promoter as previously described above.

Construction of Yarrowia lipolytica strains incapable of synthesising ergosterol

The tetrahymanol producing strain ST11005 was used for the construction of strains incapable of synthesising ergosterol. Firstly, to create a *erg5* knock-out strain, the ERG5 gene (sterol C-22 desaturase, YALI1_A18344g) was deleted by marker-mediated knock-out as described above to generate ST11014. The HphMX resistance marker was subsequently looped out by Cre-Lox

recombination to provide strain ST11027 (produced 3.1 mg/g DCW ergosta-5,7-dienol, comprising 60.0% of the total sterol fraction; the strain also produced 5.2 mg/g DCW tetrahymanol, comprising 36.7% of the total sterol fraction).

To create a ΔERG5ΔERG4 strain, the ERG4 gene (delta-24 sterol reductase, YALI1_D24361g) was deleted by HphMX marker-mediated knockout in an analogous manner. As noted above, the resulting strain ST11040 produced 3.1 mg/g DCW ergosta-5,7,24(28)-trienol, comprising 20.0% of the total sterol fraction. The strain also produced 15.8 mg/g DCW tetrahymanol, comprising 75.2% of the total sterol fraction.

Construction of strains capable of producing one or more exogenous sterols

The *ΔERG5ΔERG4* strain ST11040 was used for the construction of 24-methlyenecholesterol-producing strains. The delta-7 sterol reductase gene variant from *Tetraselmis* sp. GSL018 was expressed under the control of the PrTEFintron promoter, generating strain ST11064 as described above (produced 47.7 mg/g DCW 24-methylenecholesterol).

Strain ST11064 was then used to create strains capable of producing 24-methylenecholesterol and campesterol. The native ERG4 gene was re-introduced, under the control of previously characterised weak promoters (Holkenbrink *et al*, 2018). *ERG4* was expressed under the control of the Pr*DGA1* promoter in ST11064, generating ST11337 which produced 2.8 mg/g DCW 24-methylenecholesterol and 2.2 mg/g DCW campesterol. *ERG4* was expressed under the control of the PrGPAT promoter in ST11064, generating ST11338 which produced 5.0 mg/g DCW campesterol and trace 24-methylenecholesterol.

Strain ST11064 was also used to create a strain capable of producing 24-methylenecholesterol and campesterol by introducing the optimised coding sequence for the delta-24(28) sterol reductase of *S. tubersoum* (StSSR1) under the control of the PrDGA1 promoter. This resulted in strain ST11943 which produced 18.4.mg/g DCW 24-methylenecholesterol and 3.0 mg/g DCW campesterol (see Fig. 18A). Importantly, as further discussed below the choice of a plant delta-24(28) sterol reductase (DWF1) for expression in this strain ensured the production of the plant epimer of campesterol. Strain ST11943 was further engineered to attain additional non-native sterol mixtures.

The C-28 sterol methyl transferase from *C. quinoa* was expressed under the control of the PrGPAT promoter in strain ST11943 to enable the production of C-24 ethyl sterols (Fig 18A). The resulting strain ST12140 produced 21.4 mg/g DCW 24-methylenecholesterol, 3.7 mg/g DCW campesterol, 0.02 mg/g DCW isofucosterol, and trace amounts of beta-sitosterol.

An optimised coding sequence for the delta-24(25) sterol reductase of *S. lycopersicum* was introduced, under the control of the *PrGPAT* promoter, into strain ST12140 to generate strain ST12178 (Fig 18B). Strain ST12178 produced 22.6 mg/g DCW 24-methylenecholesterol, 2.8 mg/g DCW campesterol, 0.01 mg/g DCW isofucosterol, trace amounts of beta-sitosterol, 8.5 mg/g DCW cholesterol and 0.8 mg/g DCW desmosterol. Again importantly production of the plant epimer of campesterol was ensured but with 24-methylenecholesterol as the dominant non-native sterol. The

mix of non-native sterols observed led to this strain being further tested as a dried yeast powder for feeding honeybees following larger scale fermentation as further described below.

The same optimised coding sequence for the delta-24(25) sterol reductase gene of *S. lycopersicum* was also introduced, under the control of the *PrGPAT* promoter, into strains ST11337 and ST11338 to generate strains ST11378 and ST11340, respectively. The *S. lycopersicum* delta-24(25) sterol reductase enables the production of cholesterol from 24-methylenecholesterol (Sawai *et al.*, (2014) The Plant Cell 26: 3763-3774) as shown also by strain ST12178. Strain ST11378 produced 9.6 mg/g DCW 24-methylenecholesterol, 0.4 mg/g DCW campesterol and 0.4 mg/g DCW cholesterol. Unexpectedly, strain ST11378 also produced 0.1 mg/g DCW desmosterol. This is likely to be an intermediate or side-product arising from activity of the *S. lycopersicum* delta-24(25) sterol reductase. Strain ST11340 produced 4.6 mg/g DCW campesterol, trace 24-methylenecholesterol, 0.1 mg/g DCW cholesterol and trace desmosterol.

To create strains of mixed composition wherein campesterol is the dominant sterol, SMT (C-28 sterol methyltransferase) was expressed in ST11340, followed by deletion of *TtSTC* to remove tetrahymanol production. The C-28 sterol methyltransferase from *C. quinoa* was expressed under the control of the *PrGPAT* promoter in strain ST11340 to enable the production of C-24 ethyl sterols. The resulting strain, ST11362, produced 4.3 mg/g DCW campesterol and 0.1 mg/g DCW cholesterol as well as trace amounts of 24-methylenecholesterol, isofucosterol and beta-sitosterol. It was found possible to remove the *TtSTC* gene from that strain to create strain ST11441. The resulting strain can produce a mixture of phytosterols without a sterol surrogate. Strain ST11441 produced 11.9 mg/g DCW campesterol, 0.2 mg/g DCW 24-methylenecholesterol, 0.3 mg/g DCW cholesterol and trace amounts of desmosterol, isofucosterol and beta-sitosterol (see Fig. 15)

To create strains of mixed composition wherein 24-methylenecholesterol is the dominant sterol, *TtSTC* was deleted from ST11378 to remove tetrahymanol production and SMT was simultaneously introduced. The HphMX resistance marker was first removed from the ERG4 locus by Cre-Lox recombination. The *TtSTC* gene was then removed from strain ST11378 by homologous recombination with a cassette containing the C-28 sterol methyltransferase from *C. quinoa* under the control of the *PrGPAT* promoter and a NatMX resistance marker. The NatMX resistance marker was subsequently looped out by Cre-Lox recombination. The resulting strain ST11542 can produce a mixture of phytosterols without a sterol surrogate. Strain ST11542 produced 4.1 mg/g DCW 24-methylenecholesterol, 1.1 mg/g DCW campesterol, 0.3 mg/g DCW cholesterol, 0.01 mg/g DCW desmosterol and trace amounts of isofucosterol and beta-sitosterol (see Fig 15).

Cultivation in bioreactors

The temporal growth and sterol production of strain ST11064 was investigated by fed-batch cultivation in 250-ml Ambr ® bioreactors (Fig. 19). DO was maintained above 20%, pH kept at 5.5 and temperature at 30°C. Fermentations were carried out in duplicate. Defined medium has previously been used to reach high biomass concentrations in *Y. lipolytica* cultivation (Sáez-Sáez *et al*, 2020). Hence, the batch medium comprised mineral medium supplemented with yeast extract and citric acid. The addition of citric acid has been shown to increase production of the monoterpenoid linalool, which

may be due to the concurrent increase in cytosolic acetyl-CoA and flux though the melavonate pathway (Cao *et al*, 2017), which may also benefit sterol production. Yeast extract was used as an organic nitrogen source. Inorganic nitrogen was provided in the form of ammonium chloride since ammonium chloride was found to be better than ammonium sulphate for lipid accumulation when using glycerol as a carbon source (Back *et al*, 2016). The biomass concentration increased for the first 60 hrs of fermentation, at which point 24-methylenecholesterol accumulated during the stationary phase until ~130 hrs of cultivation. After 150 hours of cultivation, the final biomass concentration was 31 g/l, with a 24-methylenecholesterol content of 10.4 mg/g DCW.

Strains ST4842, ST11005 and ST12178 were cultivated in duplicate in 5 L bioreactors by fed-batch fermentation (Figures 20-22). DO was maintained above 20%, pH kept at 5.5 and temperature at 30°C. The batch medium comprised mineral media (without inorganic nitrogen) supplemented with yeast extract and peptone to create a rich medium to support biomass production. The feed was also enriched to maximise biomass production. The feed contained 300 g/l glucose with additional 5 g/l $MgSO_4 \cdot 7H_2O$, 30 g/l KH_2PO_4, 1% (v/v) vitamin solution, 2% (v/v) trace metal solution as described in (Sáez-Sáez *et al*, 2020). After a 24 hour batch phase, exponential feeding was initiated. After 60 hours of cultivation, the final biomass concentration was 68.8 g/l, with a total sterol content of 0.11 mg/g DCW for ST4842. For ST11005, fermentation ran for 72 hours and the final biomass concentration was 54.5 g/l, with a sterol content of 0.15 mg/g DCW. Strain ST12178 was cultivated for 84 hours, with a final biomass concentration was 49.0 g/l, with a total sterol content of 9.1 mg/g DCW comprising 6.0 mg/g DCW 24-methylenecholesterol, 0.4 mg/g DCW campesterol, 0.5 mg/g DCW isofucosterol, trace amounts of beta-sitosterol, 0.3 mg/g DCW cholesterol, 0.04 mg/g DCW desmosterol and 1.8 mg/g DCW tetrahymanol.

Discussion

Since tetrahymanol is able to substitute for ergosterol function in the cell, as previously the ergosterol biosynthesis pathway could subsequently be disrupted without compromising cell viability. The native genes *ERG4* and *ERG5* were deleted to prevent ergosterol synthesis and allow diversion of flux toward the non-native sterol structure 24-methylenecholesterol, upon introduction of a heterologous delta-7 sterol reductase. As noted above, it is anticipated that the ergosterol pathway is tightly regulated to control ergosterol concentration in the cell. This may partly be achieved by end-product feedback inhibition. This mechanism would be expected to be highly specific to ergosterol, to prevent interference by structurally similar early intermediates of the ergosterol pathway. Hence, sterol accumulation is greater in strains lacking ergosterol, particularly in those producing non-native sterols. These sterols are likely to be esterified and stored in lipid droplets to prevent toxicity.

24-methylenecholesterol is converted to campesterol by the delta-24 sterol reductase DWF1 in plants. ERG4 catalyses sterol delta-24 reduction in yeasts. Hence, the native ERG4 was re-introduced under the control of weak promoters to enable partial conversion of 24-methylenecholesterol to campesterol.

However, DWF1 in plants and ERG4 generate different epimers. The reduced 24-methyl sterol produced by a DWF1 is the C-24R epimer. ERG4 produces the C-24S epimer, as seen in ergosterol

or epi-campesterol produced previously in *Y. lipolytica* (Du et al., 2016; Zhang et al., 2017, Qian et al. 2020).

To ensure production of the C-24*R* configuration of campesterol, a plant *DWF1* was introduced under the control of a weak promoter in strain ST11064 in which the native *ERG4* was deleted. This enabled partial conversion of 24-methylenecholesterol to campesterol, rather than to C-24*S* epi-campesterol. Similarly, the C-24*R* epimer of sitosterol is produced (β-sitosterol) rather than the C-24*S* epimer (clionasterol). It is important to distinguish these isomers as the honeybee ecdysteroid hormone makisterone A is derived from campesterol. The stereochemistry at C-24 is conserved during this conversion, and the molecular implications of an alternative stereochemistry at this position are unknown.

Strains were further engineered to incorporate a C-28 sterol methyltransferase. The broad specificity of this enzyme enabled production of the 24-ethyl sterol isofucosterol. β-sitosterol is produced from isofucosterol by DWF1. A weak promoter was chosen to ensure low C-28 sterol methyl transferase activity and the production of a small amount of isofucosterol relative to 24-methylenecholesterol.

As noted above, the *S. lycopersicum* delta-24(25) sterol reductase (SSR2) has been shown to convert 24-methylenecholesterol to cholesterol (Sawai et al. 2014). Hence, Δ*ERG5*Δ*ERG4* *Y. lipolytica* yeast strains further engineered to express this plant delta-24 sterol reductase plus a delta-7 sterol reductase permit the production of a small amount of cholesterol. This is exemplified by strain ST12178 which was derived from strain ST12140 and hence additionally expressed a C-28 sterol methyltransferase. Delta-24(25) sterol reductase expression was controlled by use of a weak promoter to allow the production of a small amount of cholesterol relative to 24-methylenecholesterol. In addition, trace amounts of desmosterol were detected. This was unexpected but is expected to be an intermediate or side-product arising from activity of the *S. lycopersicum* delta-24(25) sterol reductase upon 24-methylenecholesterol.

Hence, ST12178 was considered a favoured strain for further study in the context of providing a mixed sterol containing artificial dietary composition for honeybees. It contained 24-methylenecholesterol as the dominant sterol, some campesterol as the plant epimer plus cholesterol together with low amounts of all of isofucosterol, β- sitosterol and desmosterol.

Not all strains capable of producing exogenous sterols require a tetrahymanol substitute. The *Tt*STC gene could be removed from strains ST11362 and ST11378, which could produce a mixture of exogenous sterols. Total sterol content in the resulting strains ST11441 and ST11542 is still six times greater than in the wild-type strains, suggesting that one or more of the exogenous sterols is capable of substituting for ergosterol function in the yeast cell.

Sterols accumulate during the stationary phase in yeast (Rychtera *et al*, 2010). The total nitrogen was reduced compared to standard Delft mineral medium (Jensen *et al*, 2014) in order to increase the C/N ratio, since a high C/N ratio appears to be beneficial for lipid production, (Kerkhoven *et al*, 2016; Bellou *et al*, 2016) and may therefore support the accumulation of hydrophobic sterol compounds. The C/N of the batch media in the Ambr bioreactors was approximately 15. Hence, increasing the C/N may be expected to promote lipid accumulation and increase sterol content.

For stains ST4842, ST11005 and ST12178, the final biomasses achieved in 5-L fermentation were 68.8, 54.5 and 49.0 g/L respectively. The corresponding total sterol contents were 0.1, 0.2 and 9.1 mg/g DCW. The sterol content was significantly higher in the ST12178 than either the wild-type strain or the tetrahymanol producing strain ST11005. This suggests that the ergosterol present in ST4842 and ST11005 may lead to feedback inhibition on the sterol pathway. This effect may be reduced in ST12178 that lacks the native ergosterol, allowing greater total sterol content. The biomass specific yield for the ST12178 fermentation was consistent with that obtained in small scale cultivation. However, the sterol content of ST12178 was not increased beyond the level obtained in small scale cultivation, despite the increase in biomass concentration. Higher sterol accumulation may be achieved by increasing the lipid content, although this may be at the cost of biomass production. The C/N ratio of the batch media was roughly 6. Hence, increasing the C/N may again be expected to promote lipid accumulation and increase sterol content.

A similar platform strain has also been used to produce the sesquiterpenoid abscisic acid (Arnesen *et al*, 2022). The biomass yield and abscisic acid titre obtained during bioreactor cultivation were both lower than achieved in small-scale cultivation. However, a higher specific yield was obtained in bioreactor cultivation by altering conditions such as pH and dissolved oxygen. This suggests that terpenoid production in such strains is sensitive to fermentation conditions, and these can be fine-tuned to optimise production.

The engineered *Y. lipolytica* strains discussed herein demonstrate the potential for the synthesis of a tailored mixture of non-native sterol structures with specific stereochemistry. It is expected that sterol content could be improved by further strain modification and optimisation of fermentation conditions.

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Table 18: Genomic location of integration sites (based on CLIB122 genome sequence, ASM252v1)

Name of integration site	Chromosome	Genome region (bp)
IntA_1	A	1556243-1557282
IntC_1	C	1793168-1797643
IntC_2	C	1842294-1843343
IntC_3	C	568316-569321
IntD_1	D	2192680-2193710
IntE_1	E	1722042-1723055
IntE_2	E	3758687-3759710
IntE_3	E	1845309-1846364
IntE_4	E	2881519-2882566
IntF_2	F	110095-111114
IntF_3	F	795904-796943

Table 19: Construction of *Y. lipolytica* strains ST11362 and ST11441

Strain	Sterol composition (dominant sterol underlined)
ST9100	**Ergosterol**
ST11005	**Ergosterol, tetrahymanol**
ST11027	**Ergsota-5,7-dienol, tetrahymanol**
ST11040	**Ergosta-5,7,24(28)-trienol, tetrahymanol**
ST11064	**24-methylenecholesterol, tetrahymanol**
⇓	+*ERG4* delta-24 reductase
ST11338	**24-methylenecholesterol, epicampesterol, tetrahymanol**
⇓	+*DHCR24* delta-24(25) sterol reductase
ST11340	**24-methylenecholesterol, epicampesterol, cholesterol, desmosterol, tetrahymanol**
⇓	+*SMT* C-28 sterol methyltransferase
ST11362	**24-methylenecholesterol, epicampesterol, isofucosterol, sitosterol, cholesterol, desmosterol, tetrahymanol**
⇓	-*TtSTC* squalene-tetrahymanol cyclase
ST11441	**24-methylenecholesterol, epicampesterol, isofucosterol, sitosterol, cholesterol, desmosterol**

Table 20: Construction of *Y. lipolytica* strains ST11541 and ST11542

Strain	Sterol composition (dominant sterol underlined)
ST9100	**Ergosterol**
ST11005	**Ergosterol, tetrahymanol**
ST11027	**Ergsota-5,7-dienol, tetrahymanol**
ST11040	**Ergosta-5,7,24(28)-trienol, tetrahymanol**
ST11064	**24-methylenecholesterol, tetrahymanol**
⇓	+*ERG4* delta-24 reductase
ST11337	**24-methylenecholesterol, epicampesterol, tetrahymanol**
⇓	+*DHCR24* delta-24(25) sterol reductase
ST11378	**24-methylenecholesterol, epicampesterol, cholesterol, desmosterol, tetrahymanol**
⇓	+*SMT* C-28 sterol methyltransferase
ST11541	**24-methylenecholesterol, epicampesterol, isofucosterol, sitosterol, cholesterol, desmosterol, tetrahymanol**
⇓	-*TtSTC* squalene-tetrahymanol cyclase
ST11542	**24-methylenecholesterol, epicampesterol, isofucosterol, sitosterol, cholesterol, desmosterol**

Table 21: Construction of Y. lipolytica strain ST12178

Strain	Sterol composition (dominant sterol underlined)
ST9100	**Ergosterol**
↓	+ *TtSTC* squalene-tetrahymanol cyclase
ST11005	**Ergosterol, tetrahymanol**
↓	-*ERG5* C-22 desaturase
ST11027	**Ergsota-5,7-dienol, tetrahymanol**
↓	-*ERG4* delta-24 reductase
ST11040	**Ergosta-5,7,24(28)-trienol, tetrahymanol**
↓	+ *DHCR7* delta-7 reductase
ST11064	**24-methylenecholesterol, tetrahymanol**
↓	+*StSSR1* delta-24(28) reductase
ST11943	**24-methylenecholesterol, campesterol, tetrahymanol**
↓	+*SMT* C-28 sterol methyltransferase
ST12140	**24-methylenecholesterol, campesterol, isofucosterol, sitosterol, tetrahymanol**
↓	+*DHCR24* delta-24(25) sterol reductase
ST12178	**24-methylenecholesterol, campesterol, isofucosterol, sitosterol, cholesterol, desmosterol, tetrahymanol**

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Example 5 -Testing of engineered *Yarrowia lipolytica* strains in artificial diets for managed honeybee colonies

Materials and methods

Diet Preparation

Yeast strains ST4842 (wild-type, containing ergosterol), ST11005 (platform strain engineered to produce tetrahymanol, containing ergosterol and tetrahymanol) and ST12178 (mixed sterol producing strain producing desired phytosterols and tetrahymanol) were cultivated by 5-L fed-batch fermentation as described above. At the end of fermentation, the yeast biomass was recovered from the culture by centrifugation (4000xg, 20 min) and washed with deionized water. The biomass was heat-inactivated and dried (60°C for a minimum of 24h). The dried material was ground to a fine powder and stored at -20°C until further use.

The yeast biomass cannot be subject to inactivation by autoclave or chemical treatment, as this will degrade the sterols present in the yeast. Incubation at 60°C is commonly deemed sufficient for irreversible inactivation of the yeast and heat-inactivation of GMO yeast followed by feeding the inactivated yeast to live animals is a method that has been used previously in the UK (e.g. see Goh et al., 2021). Irreversible inactivation of the yeast was confirmed by a standard colony forming unit assay. The heat-inactivated dried yeast was dissolved at 10 mg/ml in water. The suspension was plated in serial dilution (100 μl plated, of 10, 1, 0.1, 0.01 and 0.001 mg/ml suspensions) on YPD-agar (yeast extract 10 g/l, peptone 20 g/l, glucose 20g/l, Bacto agar 24 g/l) and the plates were incubated at 30°C for at least seven days. No growth of *Yarrowia lipolytica* colonies was observed. The detection limit is 1 organism/mg material or 10^6 viable organisms/kg material.

The yeast biomass was then incorporated at 20% w/w into a holidic diet designed to provide additional protein, lipid, sugar, vitamins and minerals.

Since the mixed sterol yeast strain only contained trace β-sitosterol, a small amount of commercially available phytosterol mixture including β-sitosterol (β- sitosterol, BulkSupplements) was added (0.125 % w/w). This commercially available phytosterol mixture comprised 40% β-sitosterol, 27% campesterol, 10% stigmasterol and 10 % brassicasterol. Assuming 1% w/w sterol content in the mixed sterol yeast dry powder, the sterol provided by the yeast together with the β-sitosterol provided by the commercial phytosterol mixture equated to 0.25% w/w sterol in the final diet. In the base diet control treatment, additional commercial phytosterol mixture was added to match the total level of relevant sterol in the mixed sterol yeast treatment. The phytosterol mixture was added at 0.625% w/w such that final base diet contained 0.25% w/w β-sitosterol. This allowed the effects of total sterol concentration in the diet to be distinguished from the effects of sterol composition, as the sterol content of the mixed sterol yeast ST12178 is higher than that of the wild-type or tetrahymanol producing yeast strains ST4842 and ST11005 (figs 20-22).

Thus four diet types were prepared: a ‘mixed sterol yeast’ diet containing strain ST12178, a ‘wild-type yeast’ diet containing strain ST4842, a ‘platform yeast’ diet containing strain ST11005, and a base diet control without yeast supplementation. The yeast strains were mixed with a holidic diet with

added protein, oil, sugar, minerals and vitamins. The base diet control was formulated to maintain total protein, sugar, sterol and fat content at the same level as in the yeast supplemented diets.

The diets were supplied as 50g patties to APIDEA honeybee colonies managed within a glasshouse and stored at -20°C until use.

Honeybee colonies

Honeybee colonies were maintained for three months in APIDEA hives (Styrofoam queen mating mini nucleus hives, (Paynes Southdown Bee Farms Ltd, West Sussex, UK) made up of one brood box with five frames and a top feeder with a hole for patty delivery. Hives were maintained within a closed glasshouse environment designed to negate bee escape. Hives were distributed across two rooms of the glasshouse, with varying orientation. Feeders with 30% w/v sugar solution and water were distributed inside the glasshouse and replenished as required. Regular temperature and humidity measurements were recorded on a datalogger. A misting system was installed to cool the temperatures in the glasshouse.

Initially, each APIDEA hive contained 400-500 adult bees, 2-3 frames brood/larvae/eggs and 1-2 frames of honey stores, but no bee bread. Mated queens were introduced in cages with fondant three days prior to the start of the experiment. If necessary, queens were manually released from the cages on the first day of the experiment.

At the start of the experiment, diet patties were added via the top feeder, and replaced throughout the experiment as required. Six hives were randomly assigned to each treatment group (n=6). A full assessment of the hives was conducted every 15 days. Hive weight, patty weight and 'bee seams' (one seam defined as a continuous line of bees between adjacent frames, observed upon initial hive opening) were measured. Presence of the mated queen, honey stores, eggs, larvae and capped brood were checked, and brood frames were photographed for subsequent counting. Eggs, larvae and capped brood were counted in Adobe Photoshop. Statistical analysis was performed in Microsoft Excel and R (v4.2.1).

Six days after each full assessment, hives were partially assessed with minimal disruption to the colony. Hive weight, patty weight and 'bee seams' were measured. Presence of the mated queen, honey stores, eggs, larvae and capped brood were briefly checked. On days 21 and 45, hives with low populations (less than four bee seams) were topped up with orphanized bees.

Results

Diet consumption, colony size, brood production and development were monitored over the course of three months for each treatment group. Brood at egg, larval and capped stages in each hive were counted every 15 days, to assess the ability of each treatment to support both brood production and proper development (Figures 23 and 24).

No consistent trends in egg or larvae production were found to differ between treatment groups. As such, the mixed sterol diet does not appear to confer altered laying behaviour in the queen.

Although the number of eggs and larvae did not differ significantly between treatment groups, the number of capped brood cells was significantly higher in the mixed sterol yeast diet by day 60.

Although brood production declined in all treatment groups in the last month at the end of summer, the mixed sterol yeast diet conferred greater capped brood than all other treatment groups by the end of the experiment. This demonstrates that the colonies can support continued brood development later in the season. The base diet control does not support brood development to the same extent. This diet contained 0.25% b-sitosterol, to match the total sterol in the mixed sterol yeast treatment, as well as other phytosterols including campesterol and stigmasterol. This suggests that the specific type and composition of sterols in the mixed sterol yeast diet, such as 24-methylenecholesterol, isofucosterol, cholesterol and desmosterol are necessary for proper brood development.

As well as sterols, the yeast provides beneficial nutrients such as protein, amino acids and lipids, mostly oleic acid (D9C 18:1), palmitic acid (C16:0), stearic acid (C18:0), and linoleic acid (D9,12 C18:2, Athenstaedt et al., 2006). The wild-type yeast diet containing small amounts of ergosterol did not result in reduced brood production, development or colony health compared to the base diet control. This suggests additional compounds present in the yeast powder, as prepared by the method described, do not confer toxicity to the honeybee. The tetrahymanol-producing platform strain containing small amounts of both ergosterol and tetrahymanol did not result in reduced brood production, development or colony health compared to the wild-type yeast diet. This suggests that the presence of tetrahymanol does not have adverse effects, for example by competitively inhibiting sterol uptake from the gut.

There were no significant differences in the total amount of diet delivered or consumed between treatment groups. This suggests that the effects observed were not attributed to different intake of other macronutrients such as fats or protein. The presence of yeast powder or different sterol compositions also does not prompt a change in feeding behaviour. However, there may be additional nutrients provided by the mixed sterol yeast that differ from the wild-type yeast or tetrahymanol platform strains, that contribute to the effects observed.

Overall, the results demonstrate that *Yarrowia lipolytica* engineered by the methods described to produce a plurality of key phytosterols is a suitable dietary supplement for managed honeybee colonies. The provision of a phytosterol mixture including 24-methylenecholesterol, campesterol, cholesterol, desmosterol, isofucosterol and β-sitosterol can improve the duration of brood production, as well as proper brood development, compared to a diet containing sub-optimal sterol composition. Improving the sterol content in the dry yeast powder or increasing the percentage of yeast in the final diet may further support brood production and development.

References

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Goh, S., Kolakowski, J., Holder, A., Pfuhl, M., Ngugi, D., Ballingall, K., Tombacz, K., & Werling, D. (2021). Development of a Potential Yeast-Based Vaccine Platform for Theileria parva Infection in Cattle. *Frontiers in Immunology*, *12*, 2511.

Claims:

1. An oleaginous yeast for expression of one or more heterologous genes for production of one or more desired non-native sterols or compounds derived therefrom, wherein

(i) the yeast has reduced production of ergosterol compared with a wild-type oleaginous yeast or is incapable of producing ergosterol; and

(ii) is provided with a sterol surrogate to aid cell growth.

2. An oleaginous yeast as claimed in claim 1, wherein the sterol surrogate is tetrahymanol or a hopanoid.

3. An oleaginous yeast as claimed in claim 2, wherein the oleaginous yeast comprises a heterologous nucleic acid sequence encoding a squalene-tetrahymanol cyclase or a squalene-hopene cyclase for providing the sterol surrogate, preferably wherein said heterologous nucleic acid sequence is under the control of the PrGPAT promoter or a functionally equivalent weak yeast promoter.

4. An oleaginous yeast as claimed in claim 3, wherein said heterologous nucleic acid sequence encodes *Tetrahymena thermophilia* squalene-tetrahymanol cyclase or a functional variant thereof or *Schizosaccharomyces japonicus* squalene-hopene cyclase or a functional variant thereof.

5. An oleaginous yeast as claimed in any one of claims 1 to 4, wherein the oleaginous yeast has an attenuated or deleted endogenous sterol C-22 desaturase (ERG5) or an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6)

6. An oleaginous yeast as claimed in claim 5, wherein the oleaginous yeast has an attenuated or deleted endogenous sterol C-22 desaturase (ERG5) and further comprises an attenuated or deleted endogenous delta-24 sterol reductase (ERG4) and/or sterol C-24 methyltransferase (ERG6).

7. An oleaginous yeast as claimed in any preceding claim, wherein the oleaginous yeast further comprises one or more heterologous nucleic acid sequences capable of expression to provide one or more of:

a. a delta-7 sterol reductase enzyme;
b. a delta-24(28) sterol reductase enzyme;
c. a delta-24(25) sterol reductase enzyme;
d. a sterol C-28 methyltransferase enzyme and
e. a sterol C-22 desaturase enzyme,

whereby production of said one or more desired non-native sterols or compounds derived therefrom can be achieved.

8. An oleaginous yeast as claimed in claim 7,

a. wherein the oleaginous yeast comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising campesterol;

b. wherein the oleaginous yeast comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising 24-methylenecholesterol;

c. wherein the oleaginous yeast comprises:
(i) an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6),
optionally an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or optionally an attenuated or deleted delta-24 sterol reductase enzyme (ERG4) and;
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising desmosterol;

d. wherein the oleaginous yeast comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted sterol C-24 methyltransferase (ERG6);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme; and
(iii) a heterologous nucleic acid sequence encoding a delta-24 sterol reductase enzyme,
whereby one or more non-native sterols can be produced comprising cholesterol;

e. wherein the oleaginous yeast comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and
(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme,
whereby one or more non-native sterols can be produced comprising isofucosterol (delta-24(28)-Z isomer) or fucosterol (delta-24(28)-E isomer);

f. wherein the oleaginous yeast comprises:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and
(iii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme,

whereby one or more non-native sterols can be produced comprising beta-sitosterol; or

g. wherein the oleaginous yeast further comprises:
(i) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and
(ii) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme
whereby one or more non-native sterols can be produced comprising stigmasterol, optionally
wherein the oleaginous yeast has an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or delta-24 sterol reductase enzyme (ERG4) and optionally additionally one or more further heterologous nucleic acid sequences are provided to express a plant delta-24(28) sterol reductase (DWF1) enzyme and/or sterol C-22 desaturase enzyme;

h. wherein the oleaginous yeast is capable of producing a mixture of desired non-native sterols and comprises:
(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated delta-24 sterol reductase enzyme (ERG4), preferably where the ERG5 gene is deleted and the activity of ERG4 is attenuated by provision of the same encoding sequence, or a corresponding plant delta-24(28) sterol reductase (DWF1) coding sequence, under the control of a weak promoter selected from PrDGA1 and functionally equivalent weak yeast promoters;
(ii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, optionally a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase and/or optionally additionally a heterologous nucleic acid sequence encoding a C-28 sterol methyltransferase, whereby said non-native sterol mixture can be produced.

9. An oleaginous yeast according to claim 8(h) which is capable of producing a mixture of desired sterols comprising 24-methylenecholesterol, campesterol and cholesterol, optionally together with one or more further non-native sterols in detectable amount, wherein the oleaginous yeast comprises:

(i) an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5), preferably deleted ERG5;
(ii) an attenuated delta-24 sterol reductase enzyme (ERG4) or ERG4 substituted by a plant DWF1 enzyme providing attenuated delta-24(28) sterol reductase activity, e.g. where the ERG4 gene coding sequence or plant DWF1 enzyme coding sequence is under the control of the PrDGA1 promoter or a functionally equivalent weak promoter;
(iii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. the delta-7 sterol reductase variant of *Tetraselmis* sp. GSL018, preferably under the control of a stronger promoter than employed for (ii), e.g. the PrTEFintron promoter or a functionally equivalent promoter;

(iv) a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, e.g. the delta-24(25) sterol reductase of *S. lycopersicum*, preferably under the control of the PrGPAT promoter or a functionally equivalent weak promoter

and optionally

(v) a heterologous nucleic acid sequence encoding a sterol C-28 sterol methyltransferase, e.g. the sterol C-28 sterol methyltransferase of *C. quinoa,* preferably under the control of the PrGPAT promoter or a functionally equivalent promoter,

whereby said mixture of non-native sterols can be produced.

10. An oleaginous yeast as claimed in any of claim 8(b), claim 8(h) and claim 9 which is capable of producing 24-methylenecholesterol in an amount of at least about 2-3 mg/g, preferably at least about 9-10 mg/g dry cell weight, when cultured at 30°C in yeast extract peptone dextrose (YPD) medium containing glucose and no sterol precursor.

11. An oleaginous yeast according to claim 8(a) wherein the yeast delta-24 sterol reductase enzyme (ERG4) is additionally substituted by a plant delta-24(28) sterol reductase enzyme (DWF1 enzyme) or an oleaginous yeast according to claim 8(h) or claim 9 wherein a plant DWF1 enzyme is expressed such that the oleaginous yeast can produce the plant epimer (24R) of campesterol.

12. An oleaginous yeast as claimed in any one of the preceding claims which is an engineered *Yarrowia lipolytica* strain where provision of said sterol surrogate is required to facilitate cell growth in the face of deletion of one or more of the ERG 4, ERG5 and ERG6 genes, preferably where said strain is engineered from *Y. lipolytica* ST9100 or another *Y. lipolytica* which shares all or some of the same modified genotype features as *Y. lipolytica* ST9100 compared with *Y. lipolytica* W29 strain Y-63746 (available from the ARS culture collection, NAUR and the ATCC) as the reference strain with increased synthesis of squalene or another sterol precursor or sterol pathway intermediate compared to that reference strain.

13. An oleaginous yeast according to claim 12 which is an engineered *Y. lipolytica* strain capable of producing a mixture of non-native sterols comprising 24-methylenecholesterol, campesterol as the 24R plant epimer, cholesterol, isofucosterol and desmosterol, wherein the oleaginous yeast comprises:

(i) a deleted endogenous sterol C-22 desaturase enzyme (ERG5),

(ii) ERG4 substituted by a DWF1 enzyme providing attenuated delta-24(28) sterol reductase activity, e.g. the delta-24(28) sterol reductase (DWF1) of *Solanum tuberosum*, where said DWF1 enzyme is under the control of the PrDGA1 promoter or a functionally equivalent weak promoter;

(iii) a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. the delta-7 sterol reductase of *Tetraselmis* sp. GSL018, where said delta-7 sterol reductase

is under the control of a stronger promoter than employed for (ii), e.g. the PrTEFintron promoter or a functionally equivalent promoter;

(iv) a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, e.g. the delta-24(25) sterol reductase of *S. lycopersicum*, preferably under the control of the PrGPAT promoter or a functionally equivalent weak promoter and

(v) a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase, e.g. the sterol C-28 methyltransferase of *C. quinoa,* preferably under the control of the PrGPAT promoter or a functionally equivalent promoter,

whereby said mixture of non-native sterols can be produced.

14. An engineered yeast as claimed in claim 13 which is capable of producing 24-methylenecholesterol as the dominant sterol, e.g. in an amount of at least about 20 mg/g dry cell weight when cultured at 30°C in yeast extract peptone dextrose (YPD) medium containing glucose and no sterol precursor, together with all of campesterol as the plant (24R) epimer, cholesterol, isofucosterol and desmosterol in quantifiable amount, preferably additionally with at least detectable beta-sitosterol

15. An oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:

a. 24-methylenecholesterol or a derivative thereof, the oleaginous yeast comprising:
   i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4); and
   ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme; or

b. desmosterol or a derivative thereof, the oleaginous yeast comprising:
   i. an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6), optionally an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or optionally an attenuated or deleted delta-24 sterol reductase enzyme (ERG4) and
   ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme; or

c. isofucosterol (delta-24(28)-Z isomer) and/or fucosterol (delta-24(28)-E isomer) or a derivative thereof, the oleaginous yeast comprising:
   i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4);

ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and

iii. a heterologous gene nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme; or

d. cholesterol or a derivative thereof, the oleaginous yeast comprising:

i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5), and an attenuated or deleted sterol C-24 methyltransferase (ERG6);

ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and

iii. a heterologous nucleic acid encoding a delta-24 sterol reductase enzyme; or

e. beta-sitosterol or a derivative thereof, the oleaginous yeast comprising:

i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5);

ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and

iii. a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme; or

f. stigmasterol or a derivative thereof, the oleaginous yeast comprising:

i. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme and

ii. a heterologous nucleic acid encoding a sterol C-28 methyltransferase enzyme; optionally

wherein the oleaginous yeast comprises an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/ or delta-24 sterol reductase enzyme (ERG4) and a heterologous nucleic acid encoding a sterol C-22 desaturase enzyme and optionally one or more further heterologous nucleic acid sequences are provided to express a plant delta-24(28) sterol reductase (DWF1) enzyme and /or sterol C-22 desaturase enzyme;

g. a sterol mixture, the oleaginous yeast comprising:

i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated delta-24 sterol reductase enzyme (ERG4), preferably where the ERG5 gene is deleted and the activity of ERG4 is attenuated by provision of the same encoding sequence, or a corresponding plant delta-24(28) sterol reductase (DWF1) enzyme coding sequence, under the control of a weak promoter selected from PrDGA1 and functionally equivalent weak yeast promoters;

ii. a heterologous nucleic acid encoding a delta-7 sterol reductase enzyme,

optionally a heterologous nucleic acid encoding a delta-24(25) sterol reductase and/or optionally additionally a heterologous nucleic acid encoding a C-28 sterol methyltransferase, whereby a non-native sterol mixture can be produced, preferably such that a sterol mixture is produced comprising both 24-methylenecholesterol and campesterol, optionally together with one or more further non-native sterols, e.g. cholesterol;

h. a sterol mixture, the oleaginous yeast comprising:

i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5), preferably deleted ERG5;

ii. an attenuated delta-24 sterol reductase enzyme (ERG4) or ERG4 substituted by a plant delta-24(28) sterol reductase (DWF1) enzyme providing attenuated delta-24 sterol reductase activity, e.g. where the ERG4 gene coding sequence or plant delta-24(28) sterol reductase enzyme (DWF1) coding sequence is under the control of the PrDGA1 promoter or a functionally equivalent weak promoter;

iii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, e.g. the delta-7 sterol reductase variant of *Tetraselmis* sp. GSL018, preferably under the control of a stronger promoter than employed for (ii), e.g. the PrTEFintron promoter or a functionally equivalent promoter;

iv. a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, e.g. the delta-24(25) sterol reductase of *S. lycopersicum*, preferably under the control of the PrGPAT promoter or a functionally equivalent weak promoter;

v. a heterologous nucleic acid sequence encoding a C-28 sterol methyltransferase, e.g. the C-28 sterol methyltransferase of *C. quinoa,* preferably under the control the PrGPAT promoter or a functionally equivalent promoter,

whereby a mixture of non-native sterols can be produced comprising 24-methylenecholesterol, campesterol and one or more further non-native sterols comprising cholesterol, preferably where 24-methylenecholesterol or campesterol is the dominant sterol of the mixture.

16. An oleaginous yeast as claimed in claim 15 which is an engineered *Yarrowia lipolytica.*

17. Use of an oleaginous yeast as claimed in claim 8(h), claim 9 or claim 13 which expresses a sterol surrogate as a parent strain to produce an oleaginous yeast according to claim 15(h) or claim 16, where the expression of said sterol surrogate is removed from said parent strain simultaneously with expression of all of a delta-7 sterol reductase, a delta-24(25) sterol reductase and a C-28 sterol methyltransferase plus attenuated ERG4 or substitute plant delta-24(28) sterol reductase enzyme (DWF1).

18. An oleaginous yeast as claimed in any one of claims 8-10 and 12-16 where a heterologous nucleic acid coding sequence for a delta-24(28) sterol reductase is required for production of said one

or more non-native sterols and said heterologous nucleic acid sequence is selected to encode a plant DWF1 enzyme

19. An oleaginous yeast as claimed in any one of claims 7 to 16 and 18, or obtained by the use of claim 17, which is engineered so that it produces one or more additional desired squalene-derived compounds, e.g. a terpenoid compound useful as pigment and/or a terpenoid compound useful as a scent in an artificial dietary composition e.g. an insect feed such as a bee feed.

20. A method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19, or obtained by the use of claim 17, under conditions whereby said one or more desired non-native sterols are synthesized.

21. A method as claimed in claim 20 wherein the culture medium comprises a carbon source, optionally isotopically-labelled, selected from one or more of:

glucose, fructose, sucrose, xylose, mannose, galactose, rhamnose, arabinose, one or more fatty acids, glycerol, acetate, citrate, pyruvate, starch, glycogen, amylopectin, amylose, cellulose, cellulose acetate, cellulose nitrate, hemicellulose, xylan, glucuronoxylan, arabinoxylan, glucomannan, xyloglucan, lignin, lignocellulose and/or vegetable oil, preferably glucose, and no sterol precursor is provided in the culture medium.

22. A method as claimed in claim 20 or claim 21 wherein said one or more non-native sterols are further converted in the same cells to one or more desired sterol-derived compounds, for example sterol esters.

23. A method as claimed in any one of claims 20 to 22 which further comprises the step of recovering from the cell culture said one or more desired non-native sterols and/or one or more desired sterol-derived compounds and/or one or more compounds derived from increased squalene production such as terpenoid compounds, including beta-carotene, beta-caryophyllene, beta-cryptoxanthin and linalool.

24. A method as claimed in claim 23 which further comprises converting one or more sterols thus recovered to one or more sterol-derived compounds.

25. A method as claimed in claim 23 or claim 24, wherein a hydrophobic solvent is employed to extract the one or more desired sterols or one or more sterol-derivatives from cells following saponification and/or dodecane is employed as an additive in the culture medium to facilitate sterol extraction from the cell membrane.

26. A method as claimed in any one of claims 23 to 25 which further comprises incorporating one or more recovered sterols and/or one or more recovered sterol-derived compounds, optionally

together with one or more recovered additional compounds derived from increased squalene production, into a composition selected from an artificial dietary composition, a food product, an agricultural composition, a cosmetic composition or pharmaceutical composition.

27. A method as claimed in claim 26 wherein said one or more recovered sterols incorporated into said composition comprise isofucosterol (delta-24(28)-Z isomer) or fucosterol (delta-24(28)-E isomer), preferably isofucosterol (delta-24(28)-Z isomer), said one or more recovered sterols optionally additionally comprising 24-methylenecholesterol and campesterol as the 24R plant epimer.

28. A method as claimed in any one of claims 20 to 22 which further comprises following said culturing recovering yeast cells from the culture medium as a yeast cell biomass and inactivating the yeast cells.

29. A method as claimed in claim 28 wherein said yeast cell biomass is heated at no more than 60°C to heat inactivate and dry the yeast cells.

30. A method as claimed in claim 29 which further comprises converting the dried yeast cell biomass to a powder.

31. A method as claimed in any one of claims 28 to 30 which further comprises incorporating said yeast cells or said dried yeast cell powder into a composition selected from an artificial dietary composition, a food product, an agricultural composition, a cosmetic composition or pharmaceutical composition.

32. A method as claimed in claim 31 wherein there is provided in said composition by said yeast cells or said dried yeast cell powder one or more sterols comprising isofucosterol (delta-24(28)-Z isomer) or fucosterol (delta-24(28)-E isomer), preferably isofucosterol (delta-24(28)-Z isomer), optionally together with 24-methylenecholesterol and campesterol as the 24R plant epimer.

33. A method as claimed in any one of claims 26, 27, 31 and 32 wherein said composition is an artificial dietary composition for bees or other insects or animals.

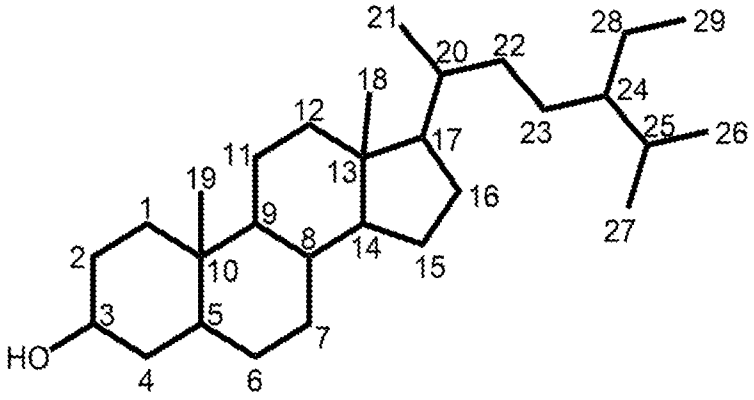
34. A method as claimed in claim 33 wherein one or more sterols are incorporated into said artificial dietary composition for bees or other insects or animals, said one or more sterols being selected from 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol produced by culturing oleaginous yeast cells according to any one of claim 8(b), claim 8(h), claim 9, claim 11 as dependent on claim 8(h) and claim 9, claim 13, claim 14, claim 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, or obtained by the use of claim 17, preferably a sterol mixture comprising both 24-methylenecholesterol and the plant epimer of campesterol, optionally together with one or more further sterols in detectable amount.

35. A method as claimed in claim 34 wherein a sterol mixture is incorporated into said artificial dietary composition which comprises 24-methylenecholesterol and isofucosterol (delta-24(28)-Z isomer), optionally together with one or more further sterols in detectable amount.

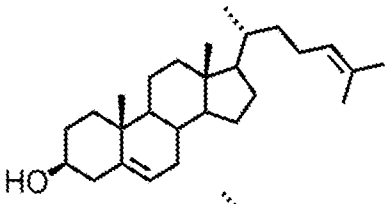
36. An artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, or obtained by the use of claim 17, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of claim 8(b), claim 8(h), claim 9, claim 11 as dependent on claim 8(h) and claim 9, claim 13, claim 14, claim 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, or obtained by the use of claim 17, recovered from a cell culture.

37. An artificial dietary composition as claimed in claim 36 wherein said recovered yeast cells are provided in the composition as a dried yeast cell powder, preferably said dried yeast cell powder comprising oleaginous yeast cells according to claim 13 or claim 14 which have been dried and converted to a powder.

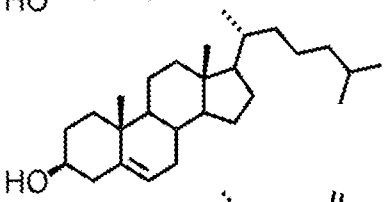
Figure 1



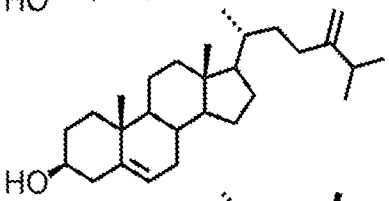
Desmosterol
Cholesta-5,24-dien-3β-ol
$C_{27}H_{44}O$



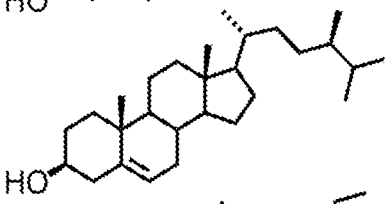
Cholesterol
Cholest-5-en-3β-ol
$C_{27}H_{46}O$



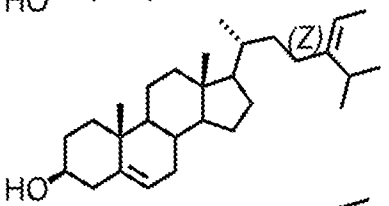
24-Methylenecholesterol
24-Methyl cholesta-5,24(28)-dien-3β-ol
$C_{28}H_{46}O$



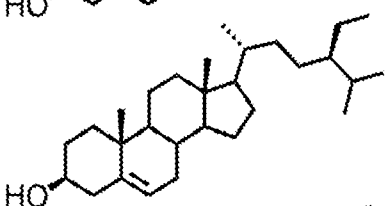
Campesterol
24α-Methyl cholest-5-en-3β-ol
$C_{28}H_{48}O$



Isofucosterol
24-Ethyl cholesta-5,24(28) trans-dien-3β-ol
$C_{29}H_{48}O$



Beta-sitosterol
24α-Ethyl cholest-5-en-3β-ol
$C_{29}H_{50}O$



Stigmasterol
24α-Ethyl cholesta-5,22 trans-dien-3β-ol
$C_{29}H_{48}O$

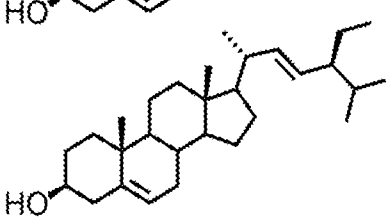


Figure 2

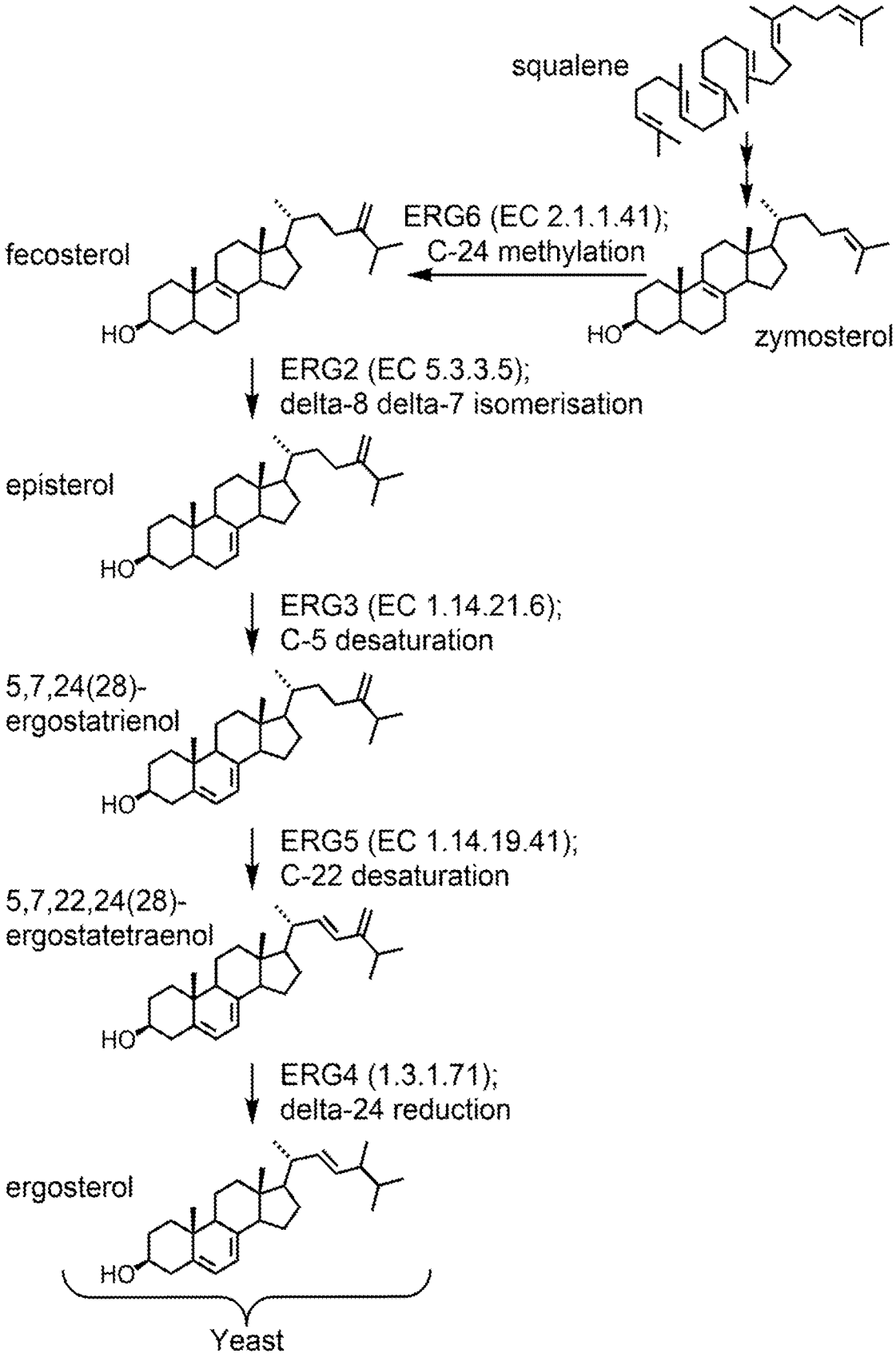

squalene
ERG6 (EC 2.1.1.41);
C-24 methylation
fecosterol
zymosterol
HO
ERG2 (EC 5.3.3.5);
delta-8 delta-7 isomerisation
episterol
ERG3 (EC 1.14.21.6);
C-5 desaturation
5,7,24(28)-
ergostatrienol
ERG5 (EC 1.14.19.41);
C-22 desaturation
5,7,22,24(28)-
ergostatetraenol
ERG4 (1.3.1.71);
delta-24 reduction
ergosterol
Yeast

Figure 3

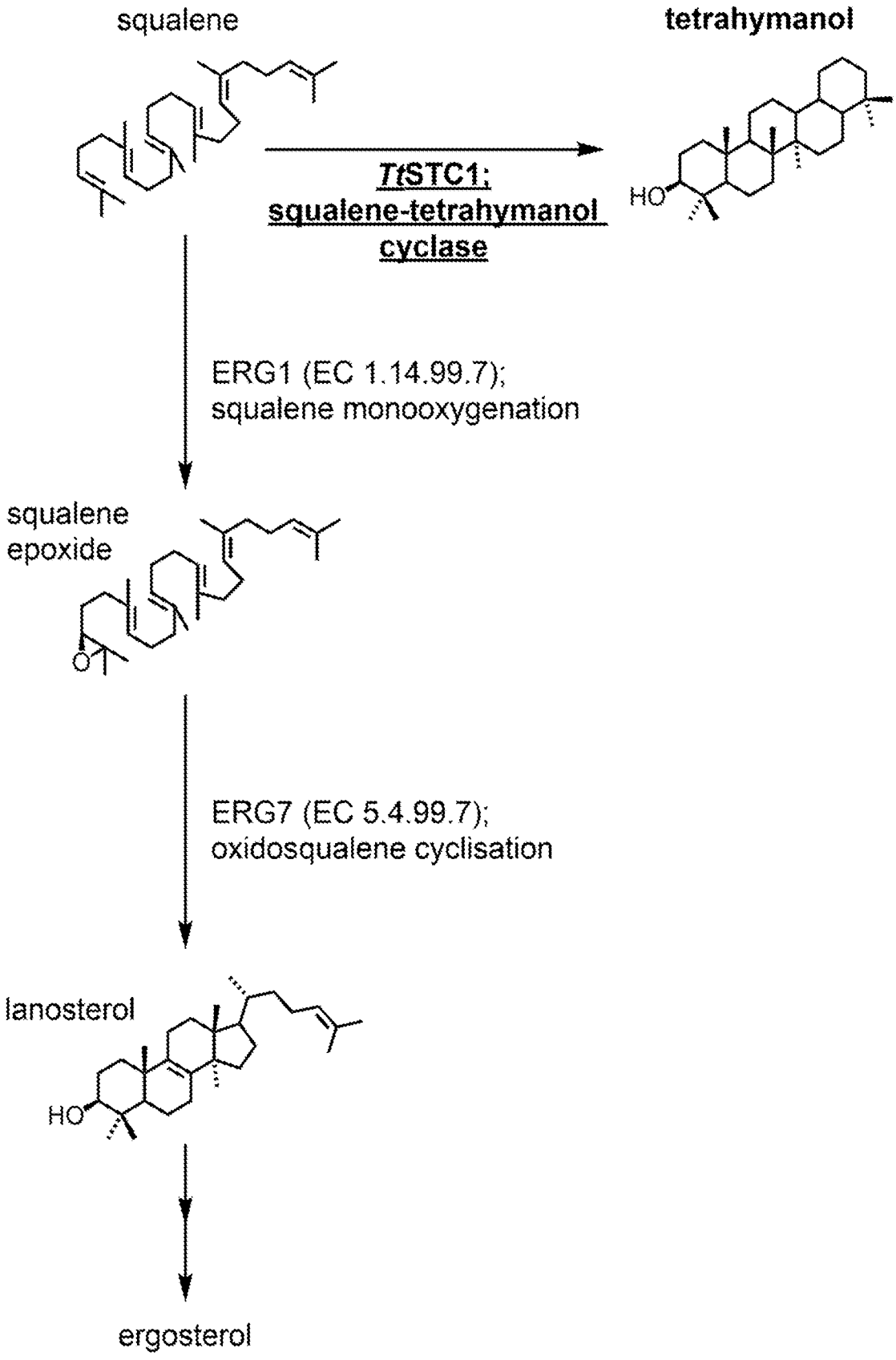

squalene
tetrahymanol
HO
*Tt*STC1;
squalene-tetrahymanol
cyclase
ERG1 (EC 1.14.99.7);
squalene monooxygenation
squalene
epoxide
O
ERG7 (EC 5.4.99.7);
oxidosqualene cyclisation
lanosterol
HO
ergosterol

Figure 4

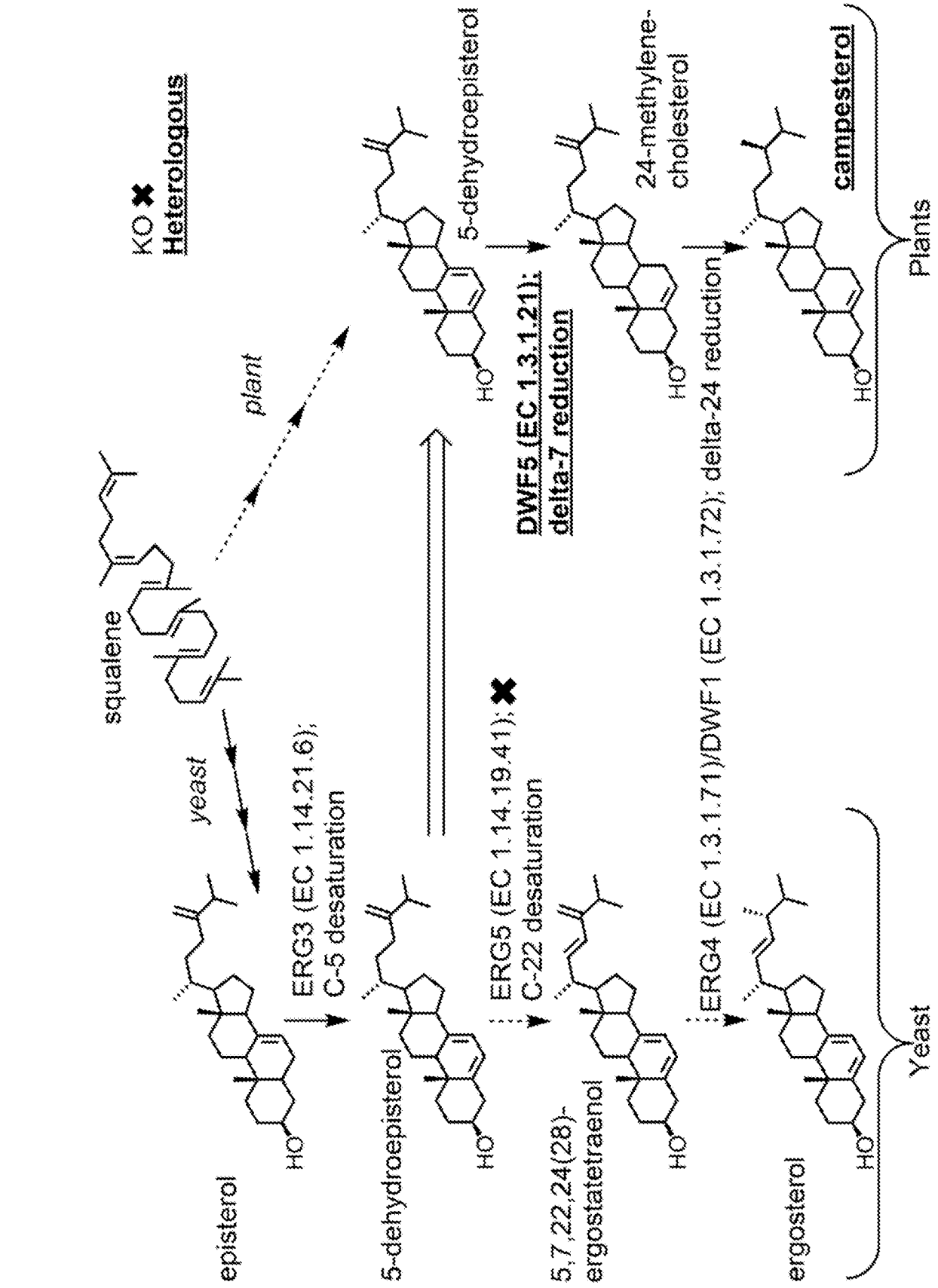

squalene
yeast
plant
KO ✖ Heterologous
episterol
ERG3 (EC 1.14.21.6); C-5 desaturation
5-dehydroepisterol
ERG5 (EC 1.14.19.41); ✖ C-22 desaturation
5,7,22,24(28)-ergostatetraenol
ERG4 (EC 1.3.1.71)/DWF1 (EC 1.3.1.72); delta-24 reduction
ergosterol
Yeast
5-dehydroepisterol
DWF5 (EC 1.3.1.21); delta-7 reduction
24-methylene-cholesterol
campesterol
Plants

Figure 5

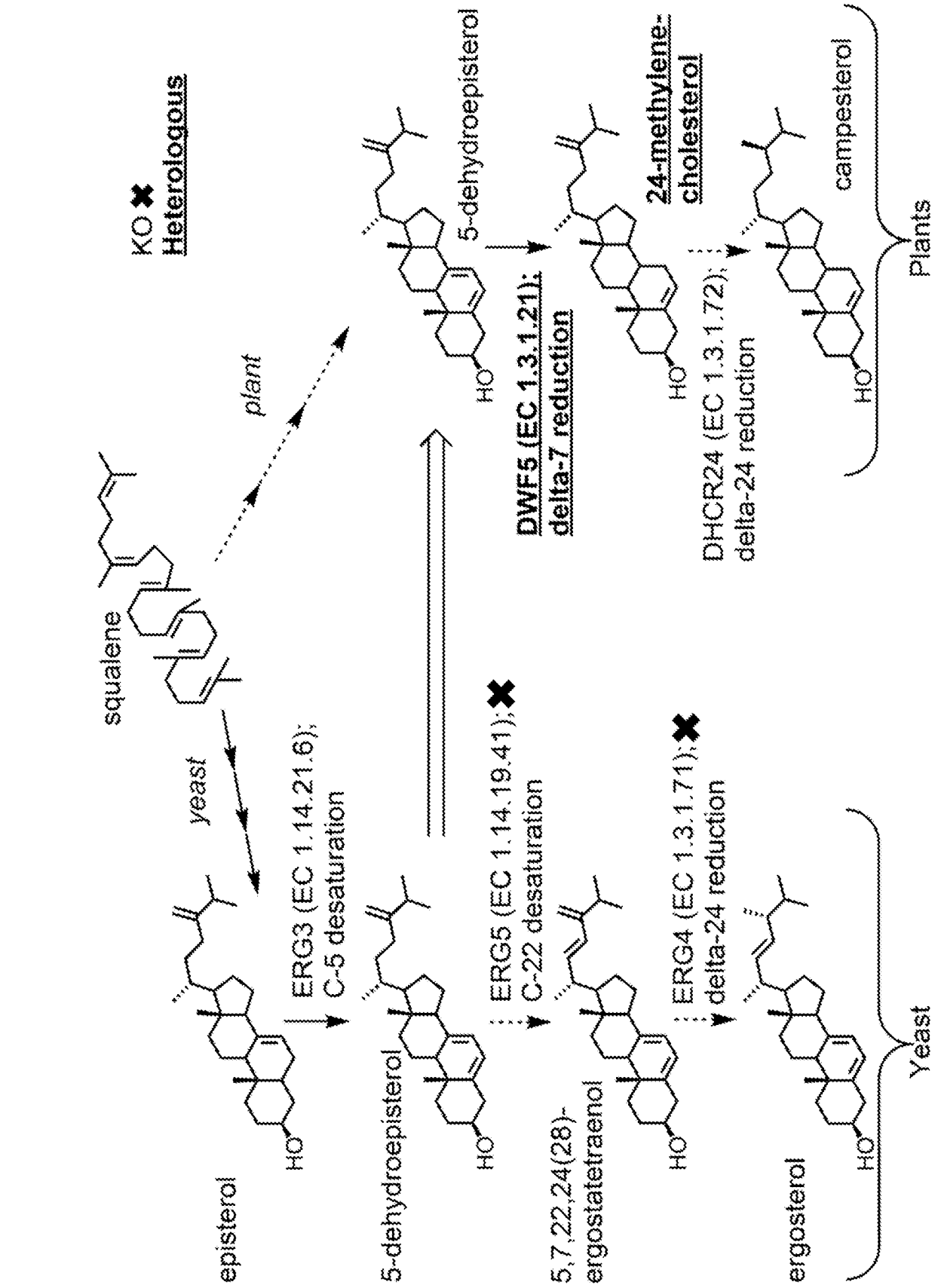

squalene
yeast
plant
KO ✖
Heterologous
ERG3 (EC 1.14.21.6);
C-5 desaturation
ERG5 (EC 1.14.19.41); ✖
C-22 desaturation
ERG4 (EC 1.3.1.71); ✖
delta-24 reduction
DWF5 (EC 1.3.1.21);
delta-7 reduction
DHCR24 (EC 1.3.1.72);
delta-24 reduction
episterol
5-dehydroepisterol
5,7,22,24(28)-ergostatetraenol
ergosterol
5-dehydroepisterol
24-methylene-cholesterol
campesterol
HO
Yeast
Plants

Figure 6

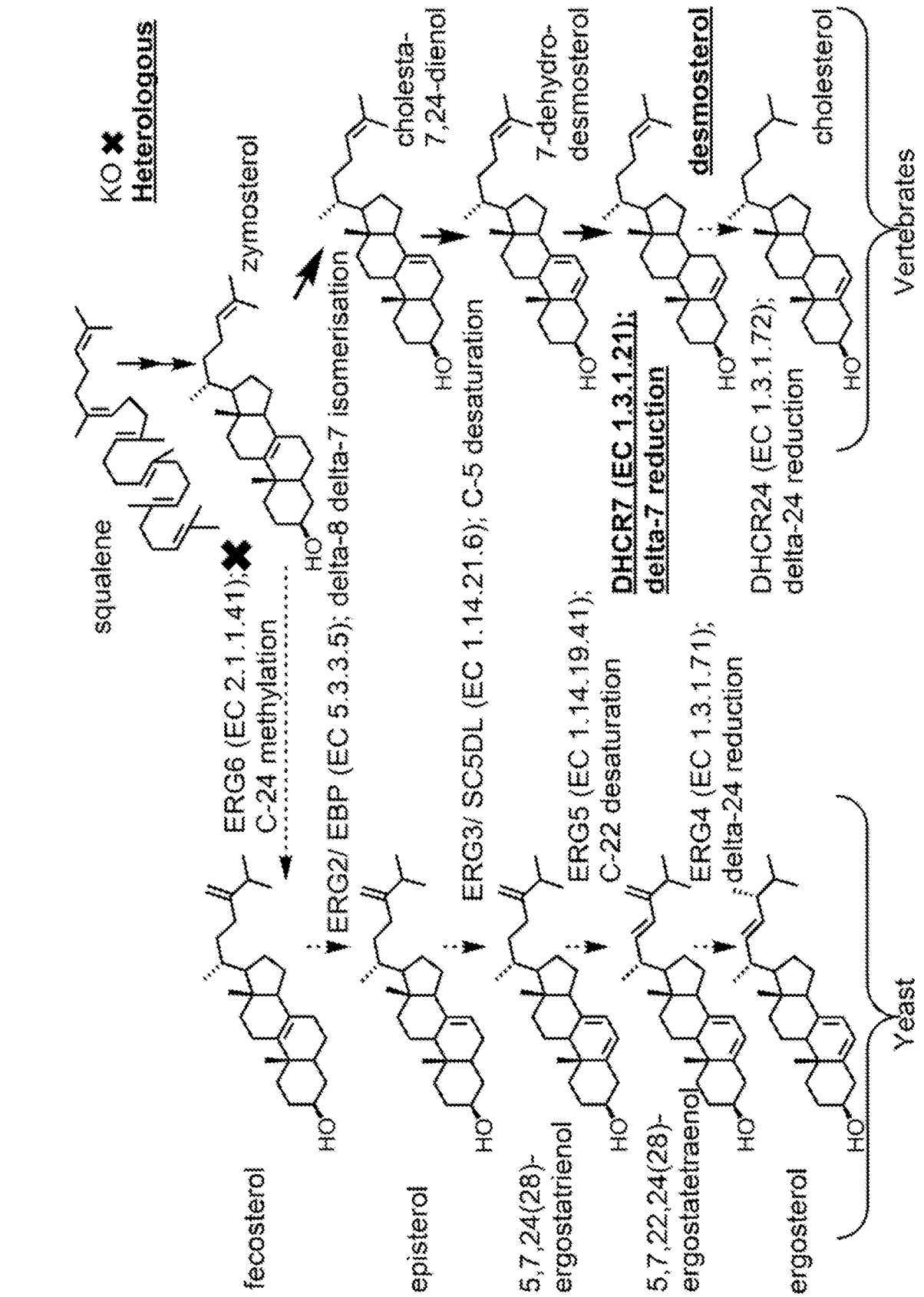

squalene
KO ✖
Heterologous
zymosterol
ERG6 (EC 2.1.1.41); C-24 methylation
ERG2/ EBP (EC 5.3.3.5); delta-8 delta-7 isomerisation
cholesta-7,24-dienol
ERG3/ SC5DL (EC 1.14.21.6); C-5 desaturation
7-dehydro-desmosterol
ERG5 (EC 1.14.19.41); C-22 desaturation
DHCR7 (EC 1.3.1.21); delta-7 reduction
desmosterol
ERG4 (EC 1.3.1.71); delta-24 reduction
DHCR24 (EC 1.3.1.72); delta-24 reduction
cholesterol
Vertebrates
fecosterol
episterol
5,7,24(28)-ergostatrienol
5,7,22,24(28)-ergostatetraenol
ergosterol
Yeast
HO

Figure 7

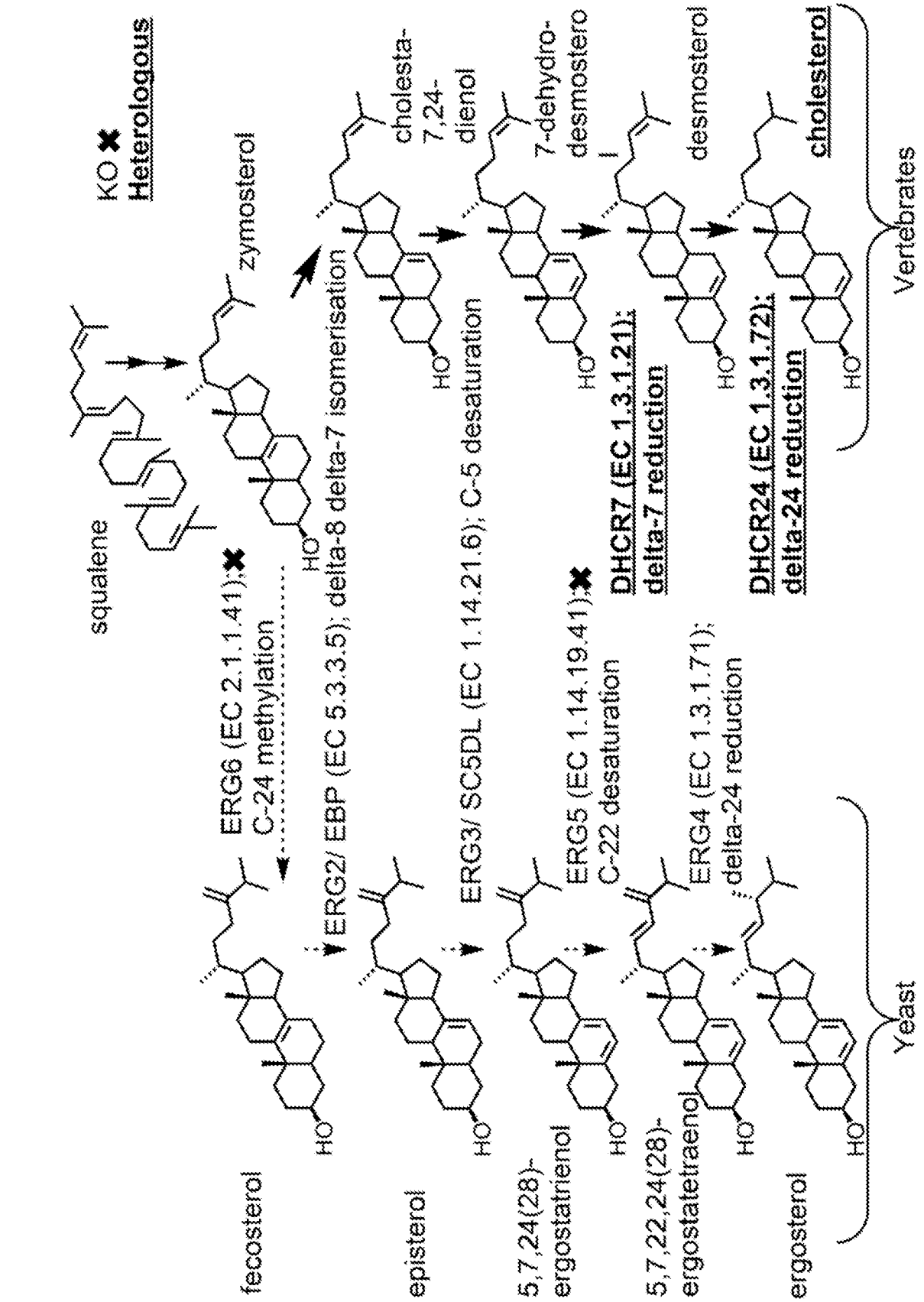

squalene
zymosterol
KO ✖ Heterologous
ERG6 (EC 2.1.1.41); ✖
C-24 methylation
ERG2/ EBP (EC 5.3.3.5); delta-8 delta-7 isomerisation
cholesta-7,24-dienol
ERG3/ SC5DL (EC 1.14.21.6); C-5 desaturation
7-dehydro-desmostero l
DHCR7 (EC 1.3.1.21); delta-7 reduction
desmosterol
DHCR24 (EC 1.3.1.72); delta-24 reduction
cholesterol
Vertebrates
ERG5 (EC 1.14.19.41); ✖
C-22 desaturation
ERG4 (EC 1.3.1.71); delta-24 reduction
fecosterol
episterol
5,7,24(28)-ergostatrienol
5,7,22,24(28)-ergostatetraenol
ergosterol
Yeast
HO

Figure 8

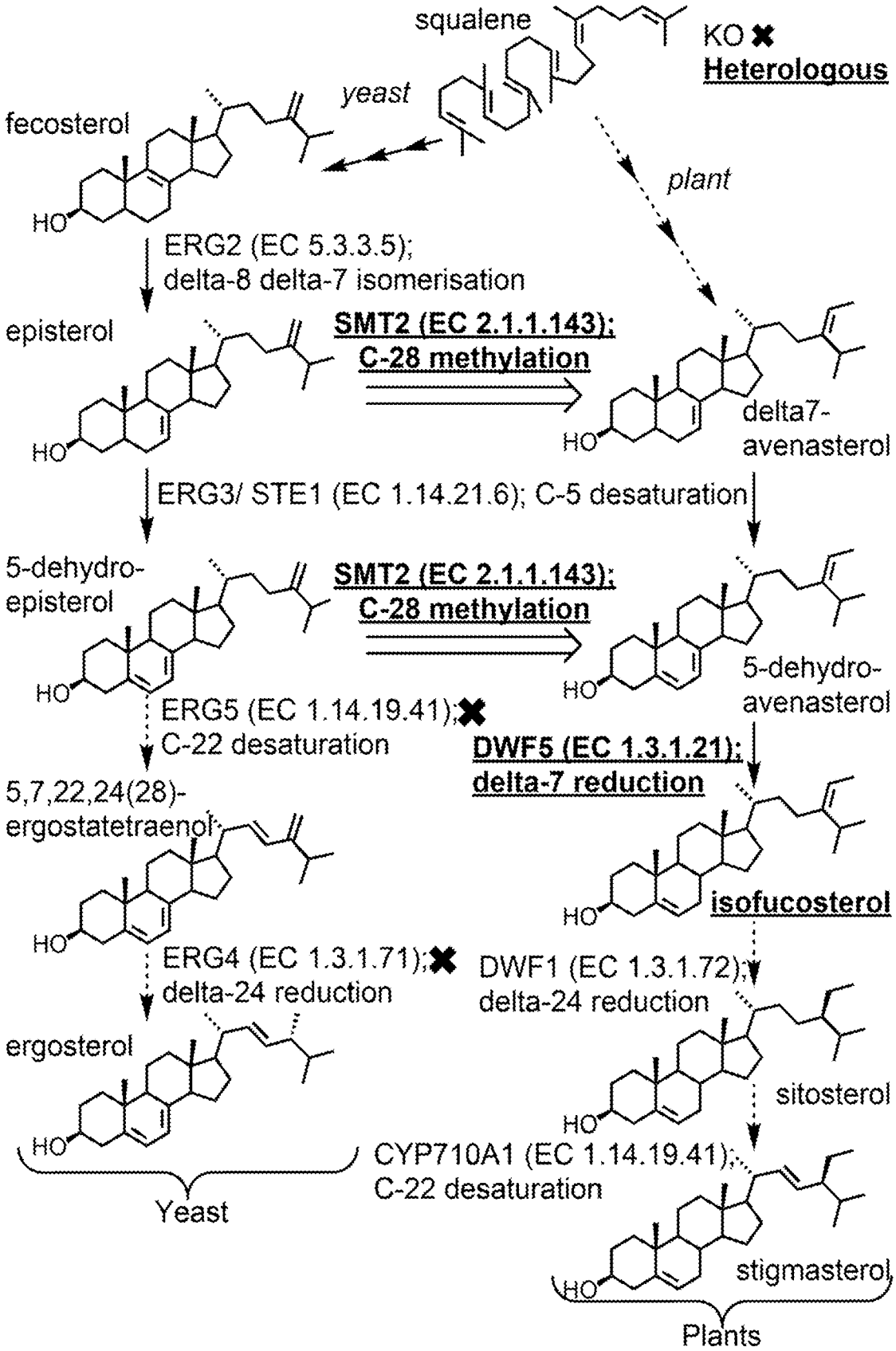

squalene
KO ✖
Heterologous
yeast
plant
fecosterol
HO
ERG2 (EC 5.3.3.5);
delta-8 delta-7 isomerisation
episterol
SMT2 (EC 2.1.1.143);
C-28 methylation
delta7-
avenasterol
ERG3/ STE1 (EC 1.14.21.6); C-5 desaturation
5-dehydro-
episterol
SMT2 (EC 2.1.1.143);
C-28 methylation
5-dehydro-
avenasterol
ERG5 (EC 1.14.19.41); ✖
C-22 desaturation
DWF5 (EC 1.3.1.21);
delta-7 reduction
5,7,22,24(28)-
ergostatetraenol
isofucosterol
ERG4 (EC 1.3.1.71); ✖
delta-24 reduction
DWF1 (EC 1.3.1.72);
delta-24 reduction
ergosterol
sitosterol
CYP710A1 (EC 1.14.19.41);
C-22 desaturation
stigmasterol
Yeast
Plants

Figure 9

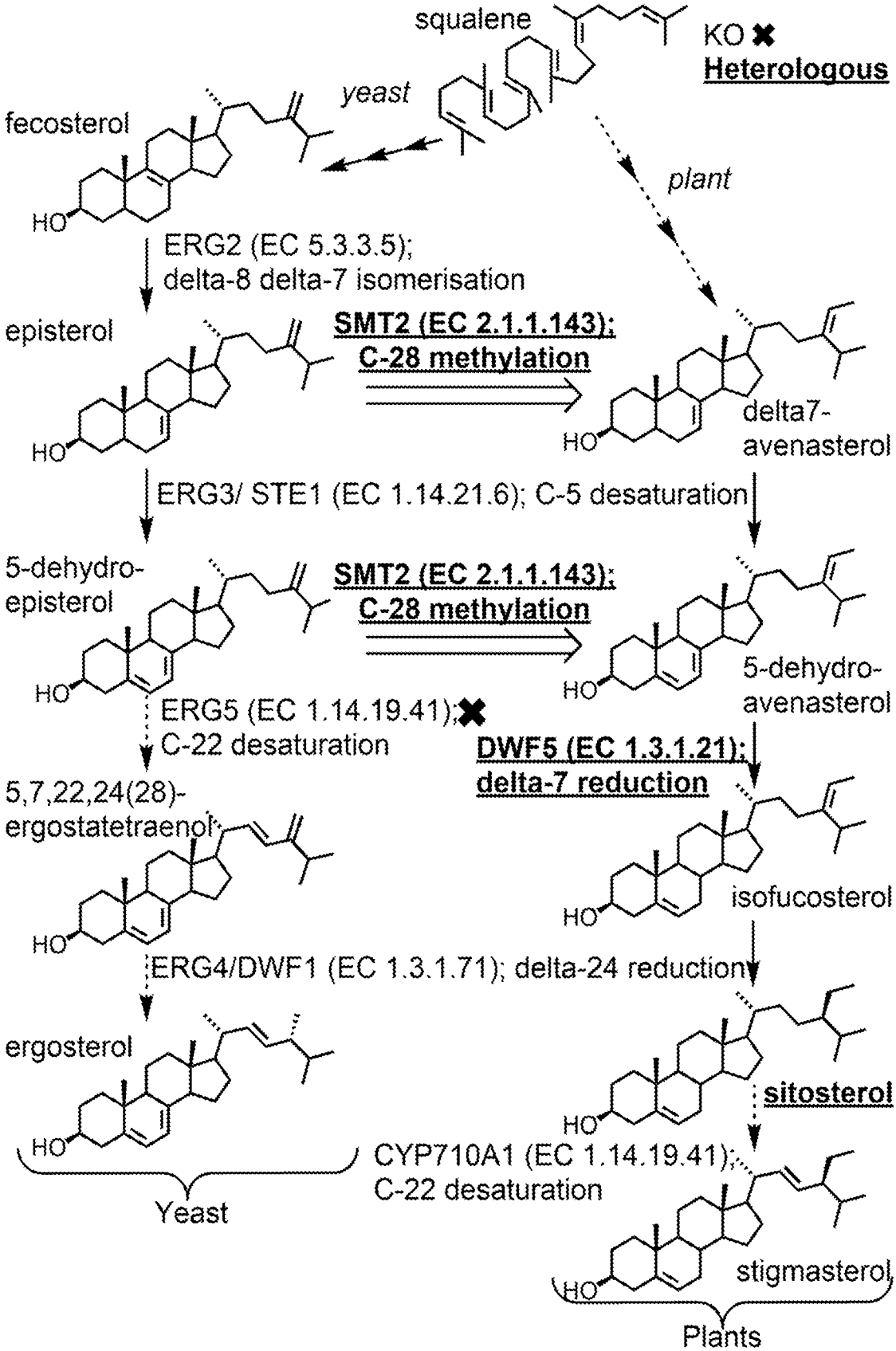

squalene
KO ✖
Heterologous
yeast
plant
fecosterol
HO
ERG2 (EC 5.3.3.5);
delta-8 delta-7 isomerisation
episterol
SMT2 (EC 2.1.1.143);
C-28 methylation
delta7-
avenasterol
ERG3/ STE1 (EC 1.14.21.6); C-5 desaturation
5-dehydro-
episterol
SMT2 (EC 2.1.1.143);
C-28 methylation
5-dehydro-
avenasterol
ERG5 (EC 1.14.19.41); ✖
C-22 desaturation
DWF5 (EC 1.3.1.21);
delta-7 reduction
5,7,22,24(28)-
ergostatetraenol
isofucosterol
ERG4/DWF1 (EC 1.3.1.71); delta-24 reduction
ergosterol
sitosterol
CYP710A1 (EC 1.14.19.41);
C-22 desaturation
stigmasterol
Yeast
Plants

Figure 10

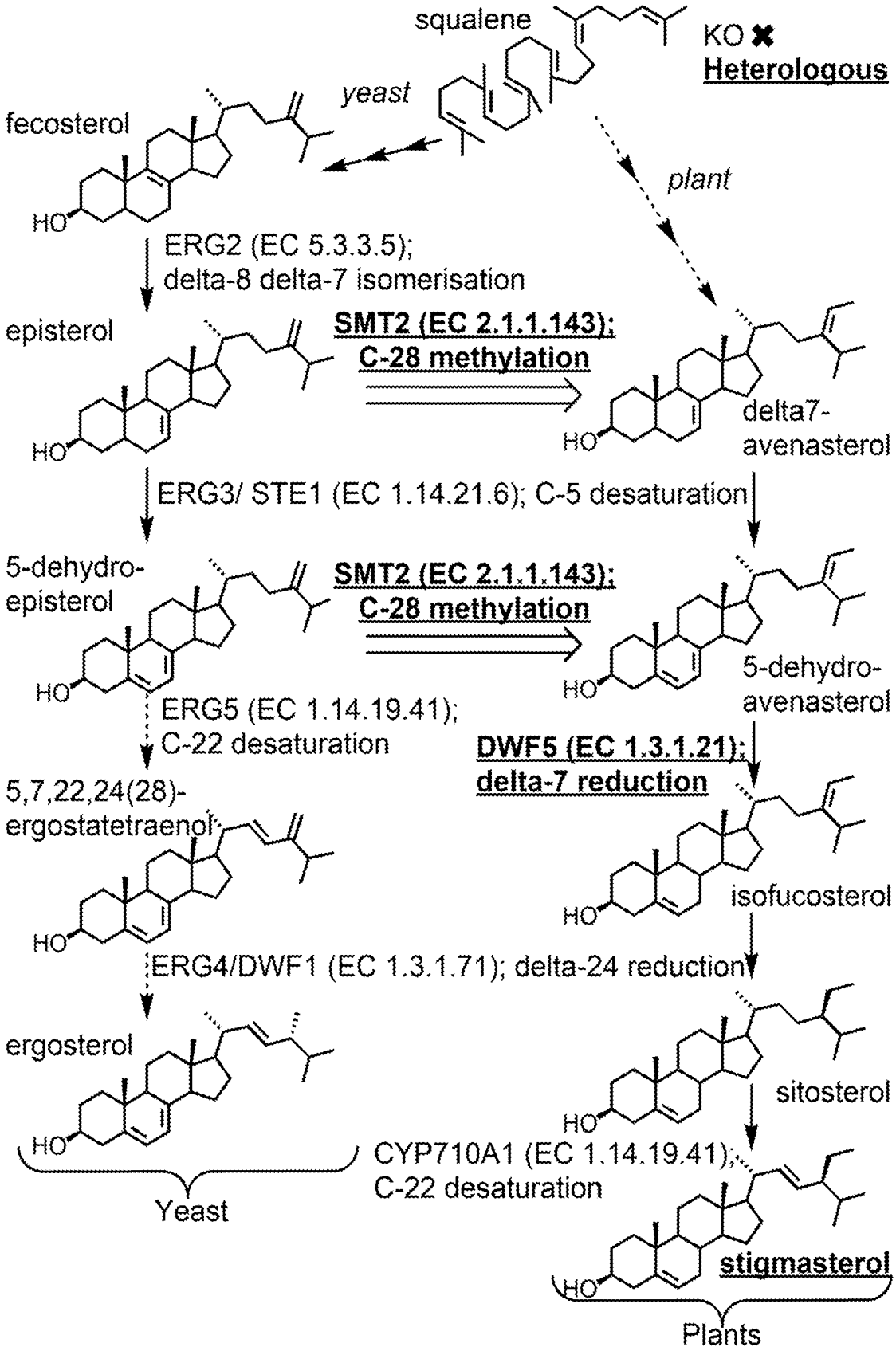

squalene
KO ✖
Heterologous
yeast
plant
fecosterol
HO
ERG2 (EC 5.3.3.5);
delta-8 delta-7 isomerisation
episterol
SMT2 (EC 2.1.1.143);
C-28 methylation
delta7-
avenasterol
ERG3/ STE1 (EC 1.14.21.6); C-5 desaturation
5-dehydro-
episterol
SMT2 (EC 2.1.1.143);
C-28 methylation
5-dehydro-
avenasterol
ERG5 (EC 1.14.19.41);
C-22 desaturation
DWF5 (EC 1.3.1.21);
delta-7 reduction
5,7,22,24(28)-
ergostatetraenol
isofucosterol
ERG4/DWF1 (EC 1.3.1.71); delta-24 reduction
ergosterol
sitosterol
CYP710A1 (EC 1.14.19.41);
C-22 desaturation
Yeast
stigmasterol
Plants

Figure 11A

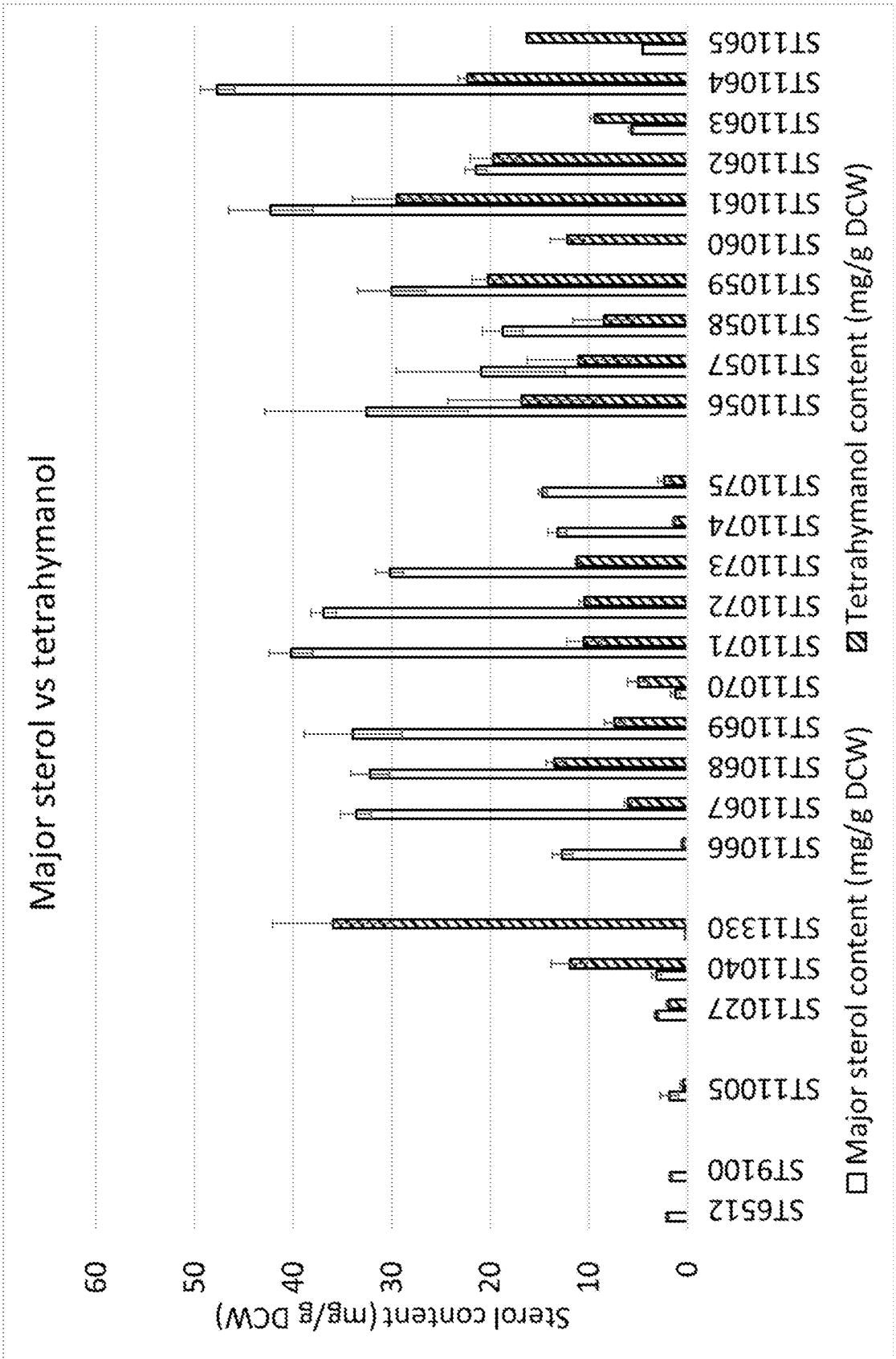

Major sterol vs tetrahymanol
Sterol content (mg/g DCW)
0
10
20
30
40
50
60
ST6512
ST9100
ST110005
ST110027
ST110040
ST113330
ST110066
ST110067
ST110068
ST110069
ST110070
ST110071
ST110072
ST110073
ST110074
ST110075
ST110056
ST110057
ST110058
ST110059
ST110060
ST110061
ST110062
ST110063
ST110064
ST110065
Major sterol content (mg/g DCW)
Tetrahymanol content (mg/g DCW)

Figure 11B

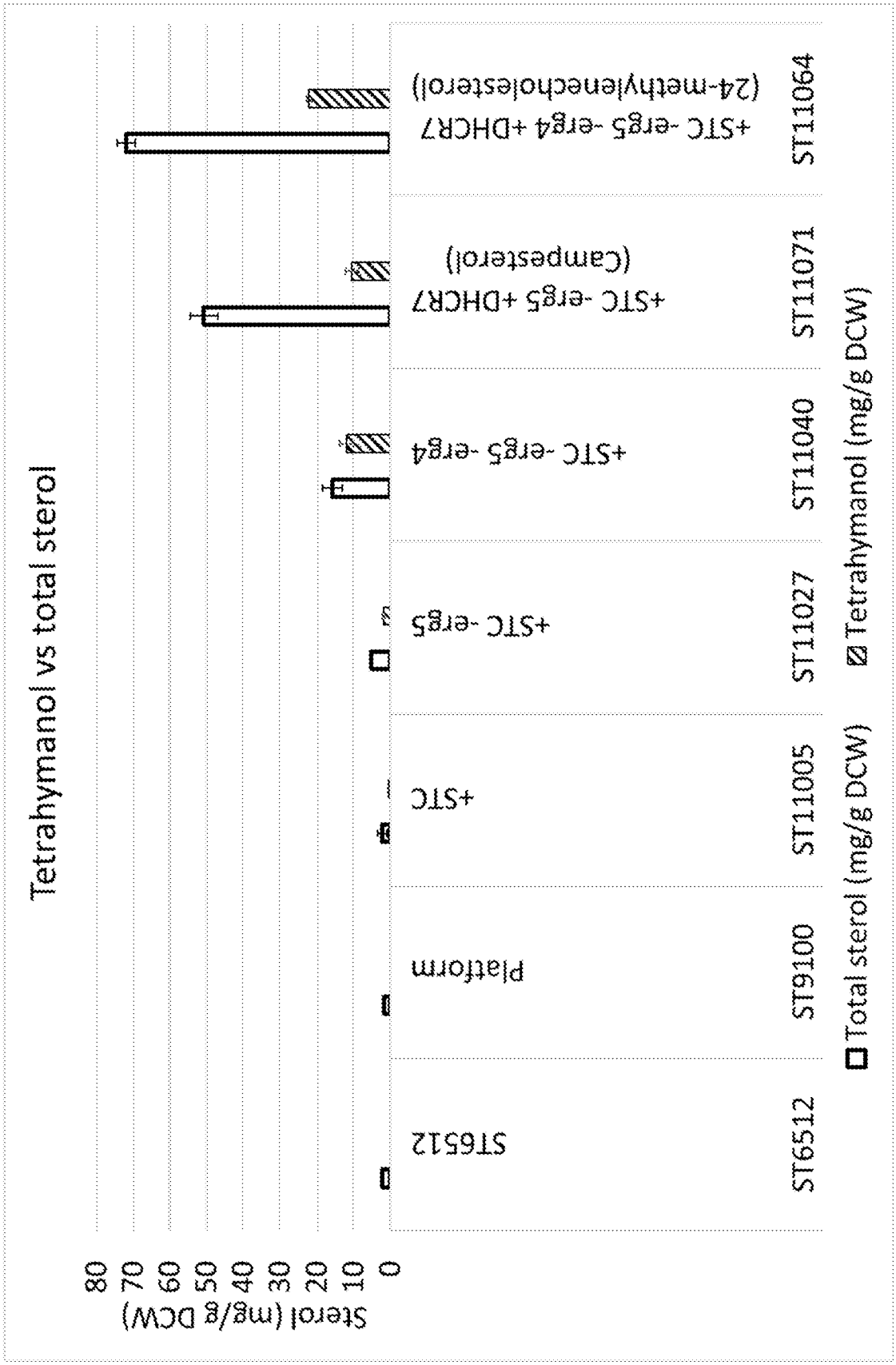

Tetrahymanol vs total sterol
Sterol (mg/g DCW)
0
10
20
30
40
50
60
70
80
ST6512
ST9100
ST11005
ST11027
ST11040
ST11071
ST11064
ST6512
Platform
+STC
+STC -erg5
+STC -erg5 -erg4
+STC -erg5 +DHCR7 (Campesterol)
+STC -erg5 -erg4 +DHCR7 (24-methylenecholesterol)
Total sterol (mg/g DCW)
Tetrahymanol (mg/g DCW)

Figure 12

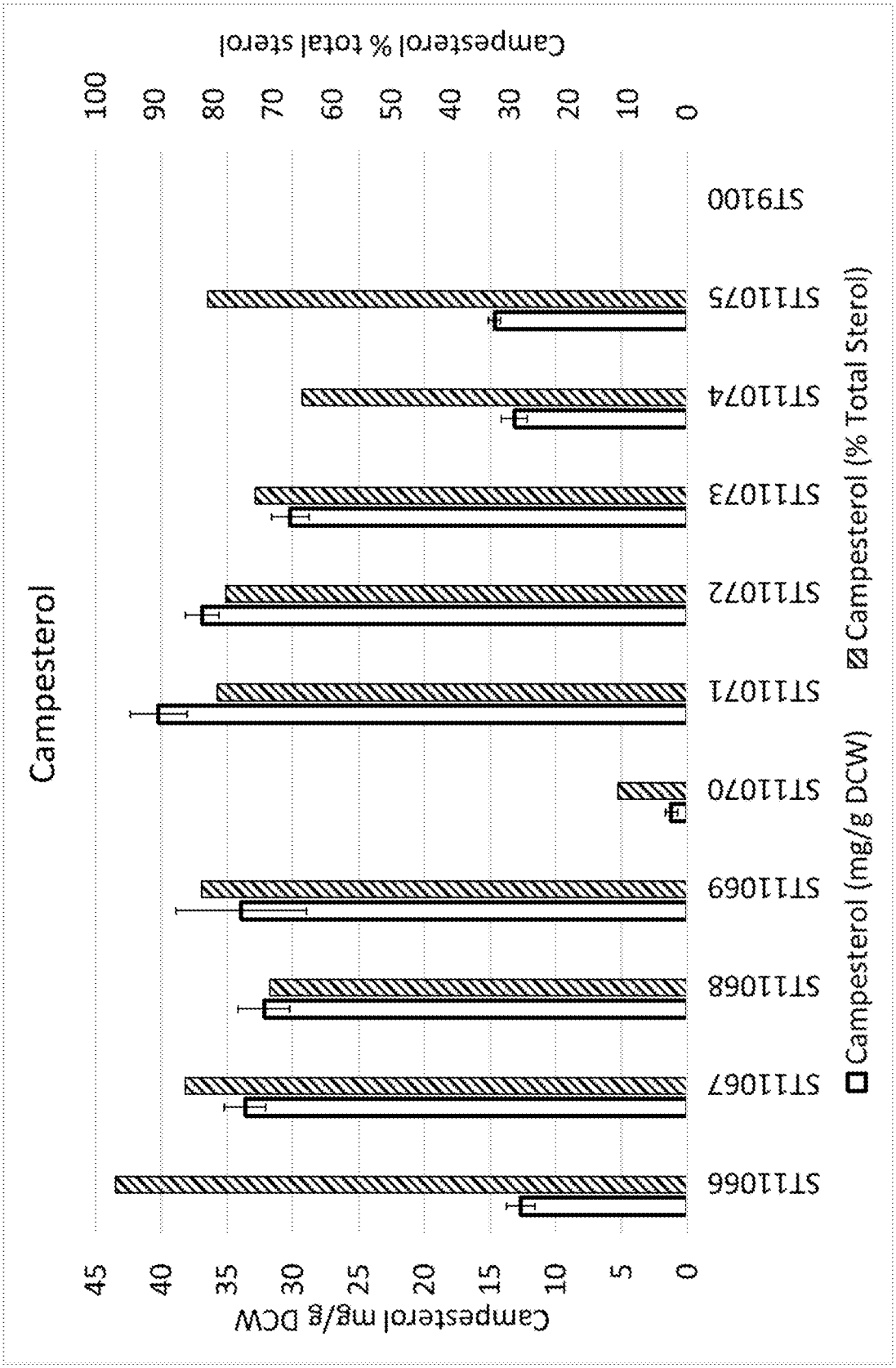

Campesterol
Campesterol mg/g DCW
Campesterol % total sterol
Campesterol (mg/g DCW)
Campesterol (% Total Sterol)
ST1106
ST1107
ST1106
ST1106
ST1106
ST1107
ST1107
ST1107
ST1107
ST1107
ST9100

Figure 13

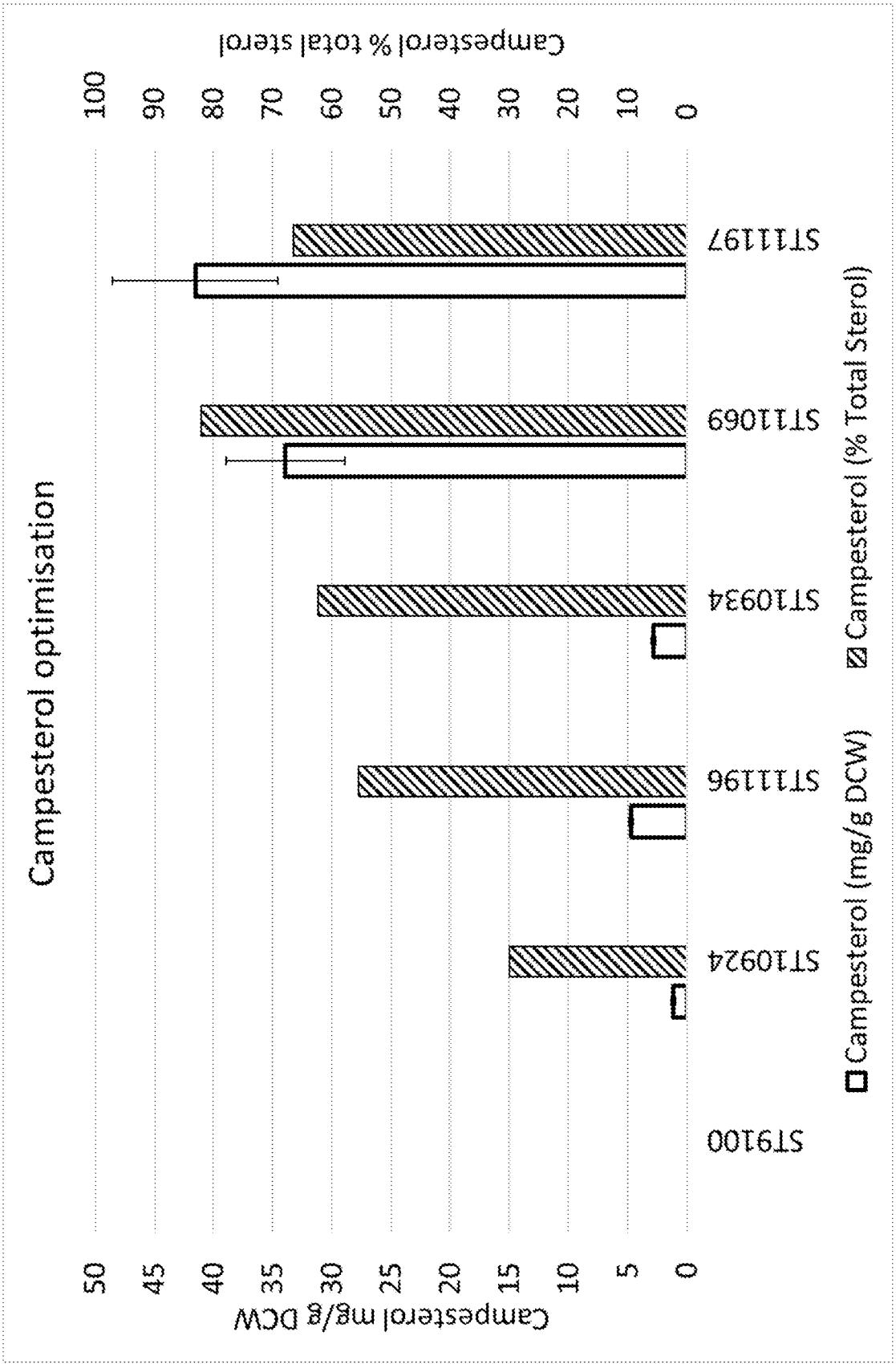

Campesterol optimisation
Campesterol mg/g DCW
50
45
40
35
30
25
20
15
10
5
0
Campesterol % total sterol
100
90
80
70
60
50
40
30
20
10
0
ST9100
ST10924
ST11196
ST10934
ST11069
ST11197
Campesterol (mg/g DCW)
Campesterol (% Total Sterol)

Figure 14

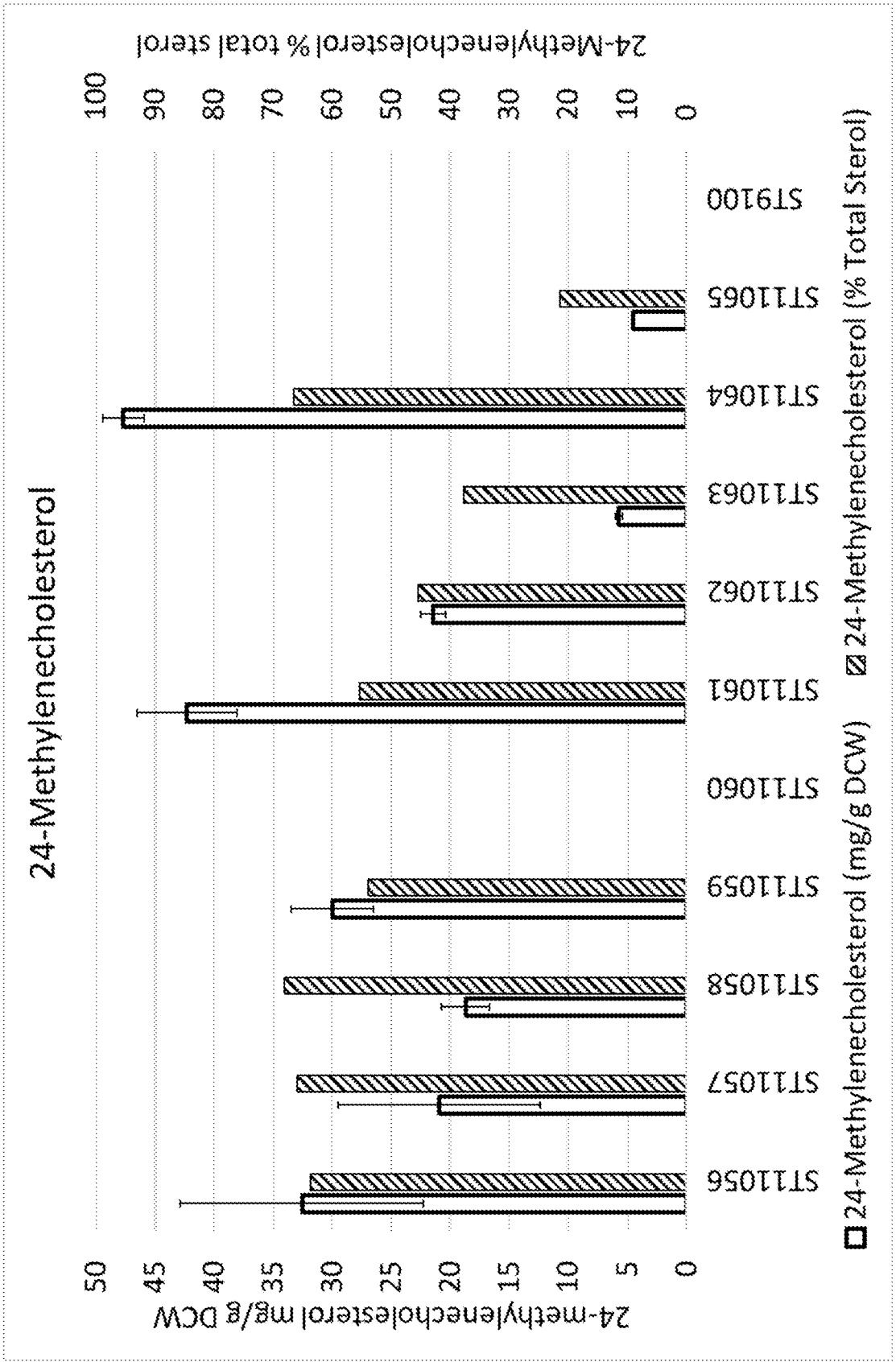

24-Methylenecholesterol
24-methylenecholesterol mg/g DCW
24-Methylenecholesterol % total sterol
24-Methylenecholesterol (mg/g DCW)
24-Methylenecholesterol (% Total Sterol)
ST11056
ST11057
ST11058
ST11059
ST11060
ST11061
ST11062
ST11063
ST11064
ST11065
ST9100
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50
60
70
80
90
100

Figure 15A

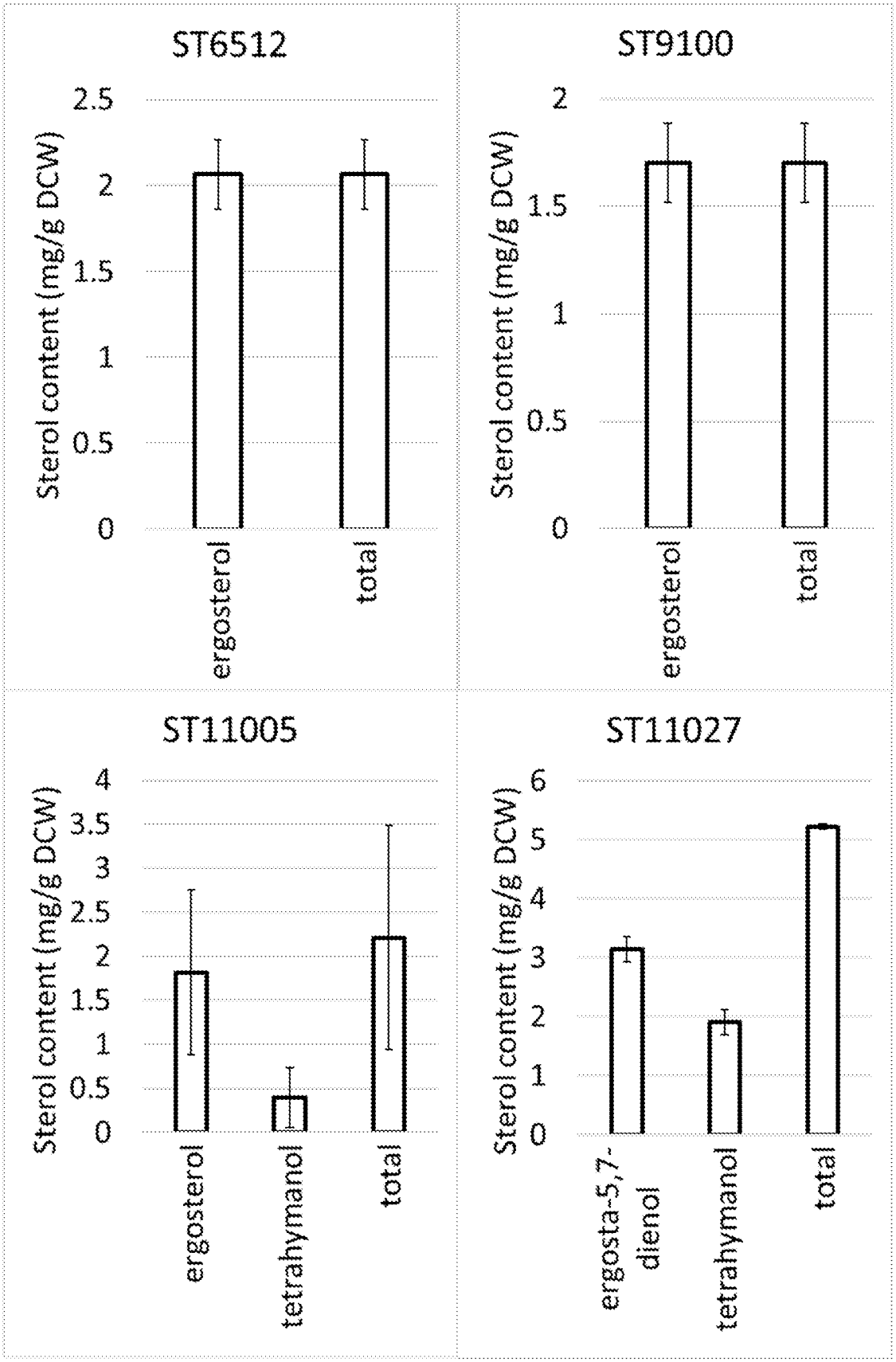

ST6512
Sterol content (mg/g DCW)
2.5
2
1.5
1
0.5
0
ergosterol
total
ST9100
Sterol content (mg/g DCW)
2
1.5
1
0.5
0
ergosterol
total
ST11005
Sterol content (mg/g DCW)
4
3.5
3
2.5
2
1.5
1
0.5
0
ergosterol
tetrahymanol
total
ST11027
Sterol content (mg/g DCW)
6
5
4
3
2
1
0
ergosta-5,7-dienol
tetrahymanol
total

Figure 15B

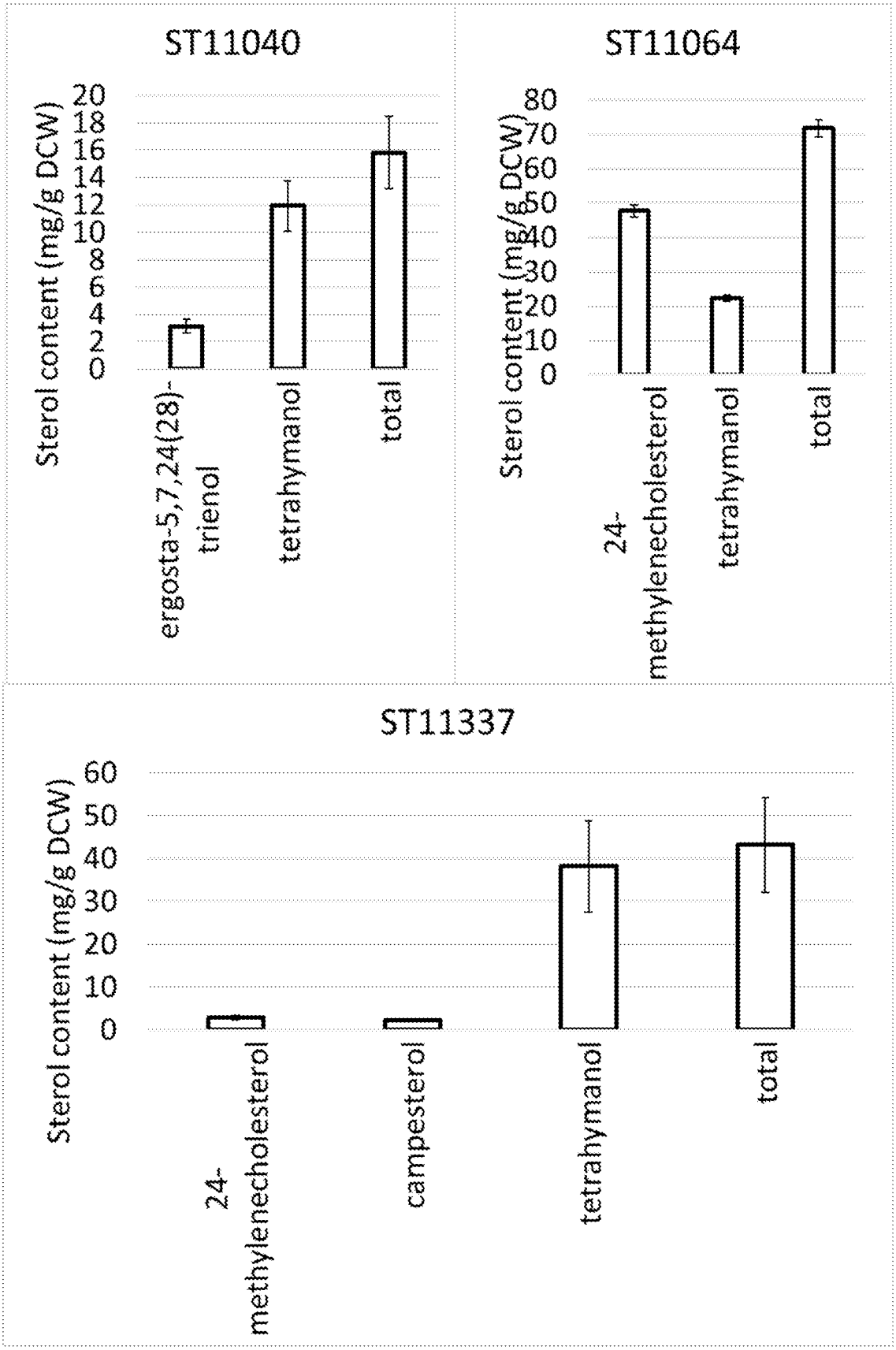

ST11040
Sterol content (mg/g DCW)
0
2
4
6
8
10
12
14
16
18
20
ergosta-5,7,24(28)-trienol
tetrahymanol
total
ST11064
Sterol content (mg/g DCW)
0
10
20
30
40
50
60
70
80
24-methylenecholesterol
tetrahymanol
total
ST11337
Sterol content (mg/g DCW)
0
10
20
30
40
50
60
24-methylenecholesterol
campesterol
tetrahymanol
total

Figure 15C

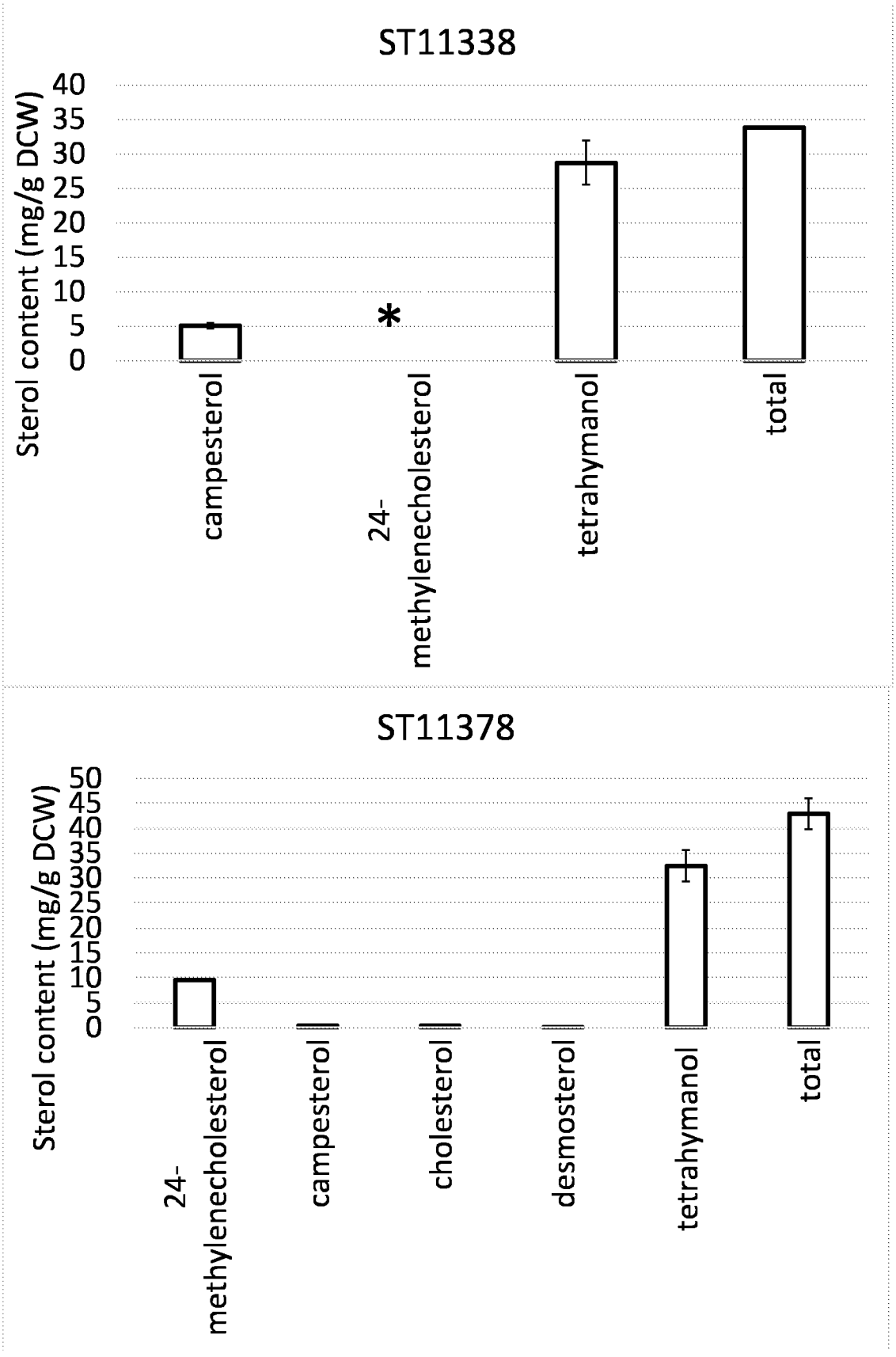

ST11338
Sterol content (mg/g DCW)
40
35
30
25
20
15
10
5
0
*
campesterol
24-methylenecholesterol
tetrahymanol
total
ST11378
Sterol content (mg/g DCW)
50
45
40
35
30
25
20
15
10
5
0
24-methylenecholesterol
campesterol
cholesterol
desmosterol
tetrahymanol
total

Figure 15D

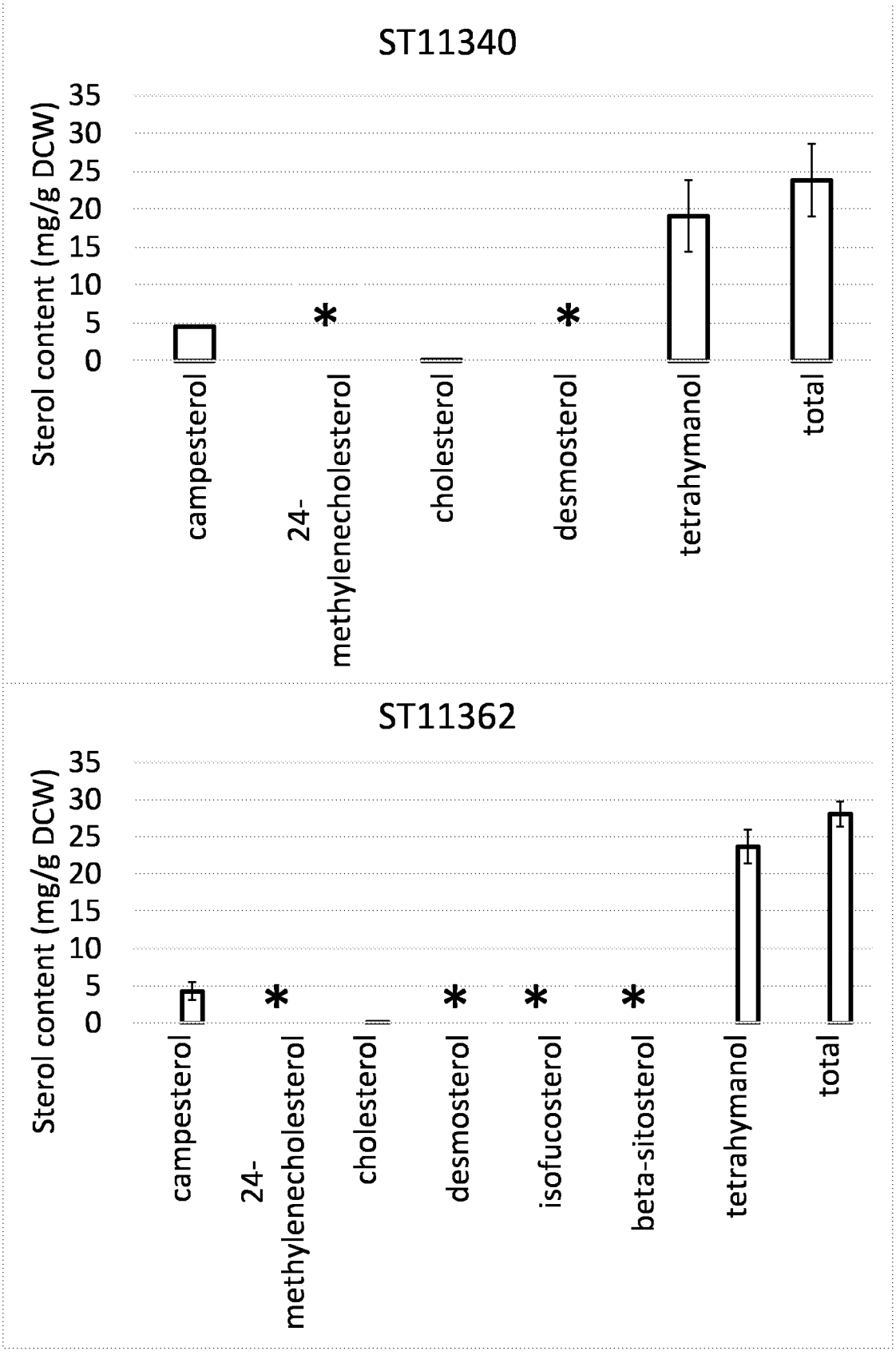

ST11340
Sterol content (mg/g DCW)
35
30
25
20
15
10
5
0
*
*
campesterol
24-methylenecholesterol
cholesterol
desmosterol
tetrahymanol
total
ST11362
Sterol content (mg/g DCW)
35
30
25
20
15
10
5
0
*
*
*
*
campesterol
24-methylenecholesterol
cholesterol
desmosterol
isofucosterol
beta-sitosterol
tetrahymanol
total

Figure 15E

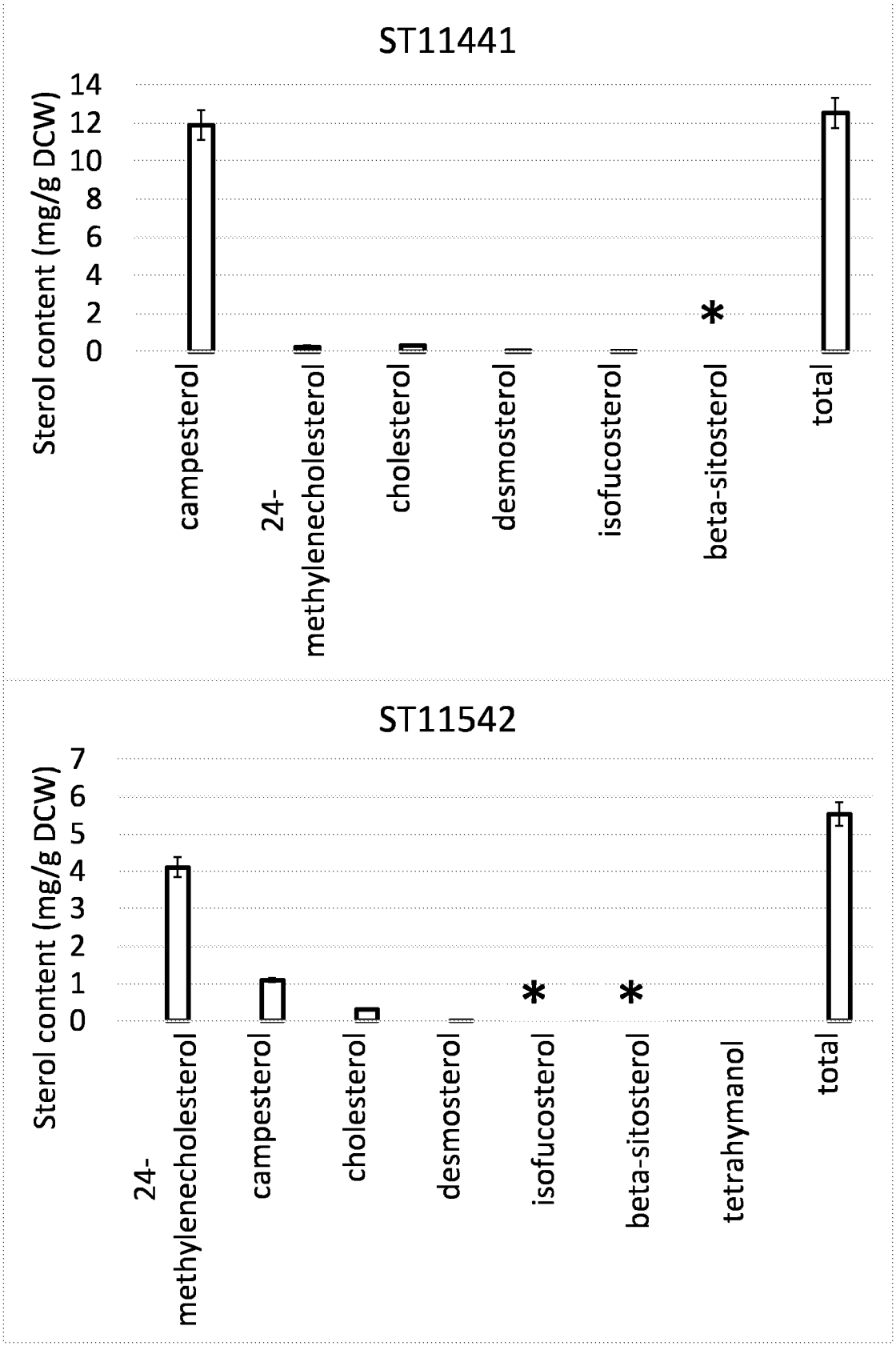

ST11441
Sterol content (mg/g DCW)
14
12
10
8
6
4
2
0
campesterol
24-methylenecholesterol
cholesterol
desmosterol
isofucosterol
beta-sitosterol
total
*
ST11542
Sterol content (mg/g DCW)
7
6
5
4
3
2
1
0
24-methylenecholesterol
campesterol
cholesterol
desmosterol
isofucosterol
beta-sitosterol
tetrahymanol
total
*
*

Figure 16A

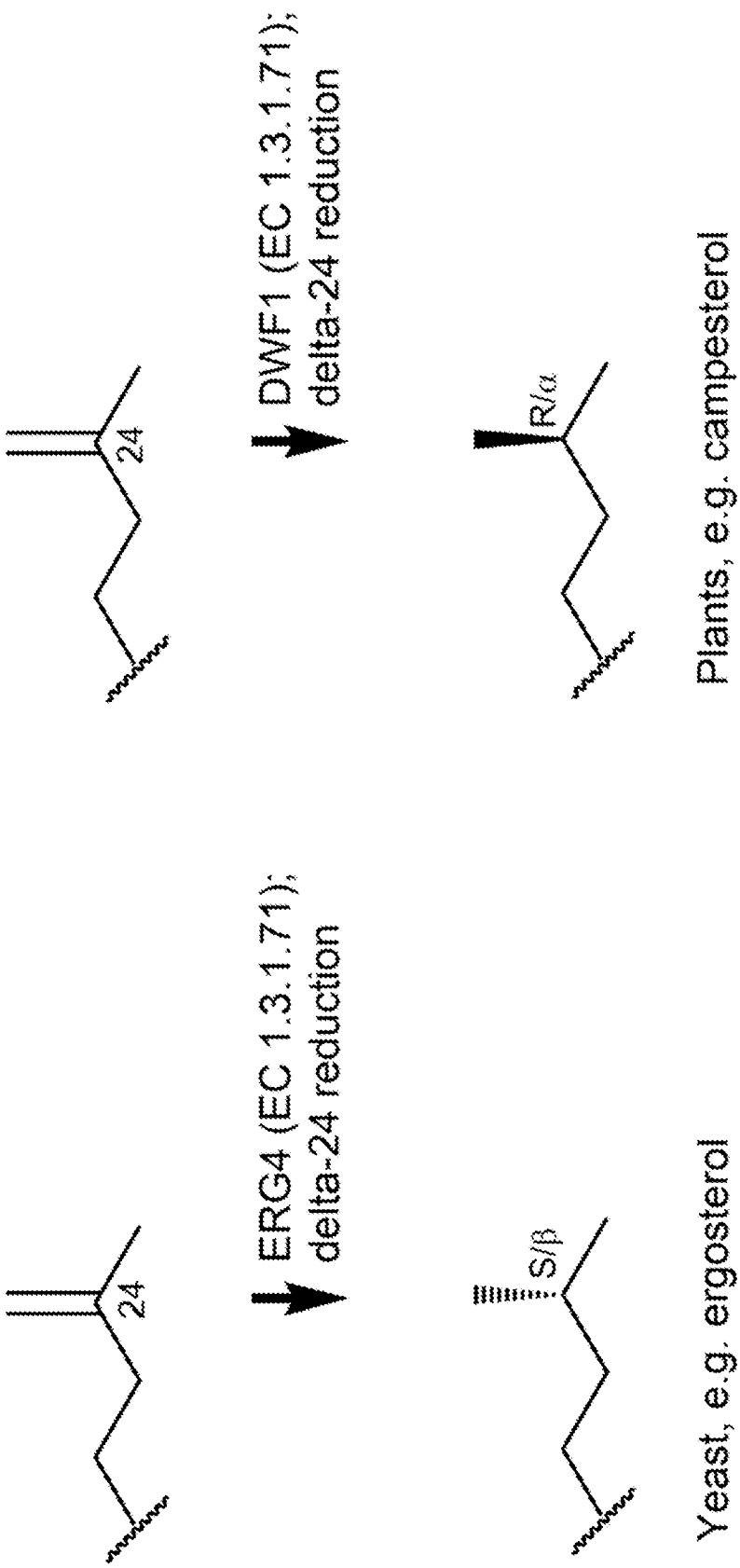

24
DWF1 (EC 1.3.1.71);
delta-24 reduction
R/α
Plants, e.g. campesterol
24
ERG4 (EC 1.3.1.71);
delta-24 reduction
S/β
Yeast, e.g. ergosterol

Figure 16B

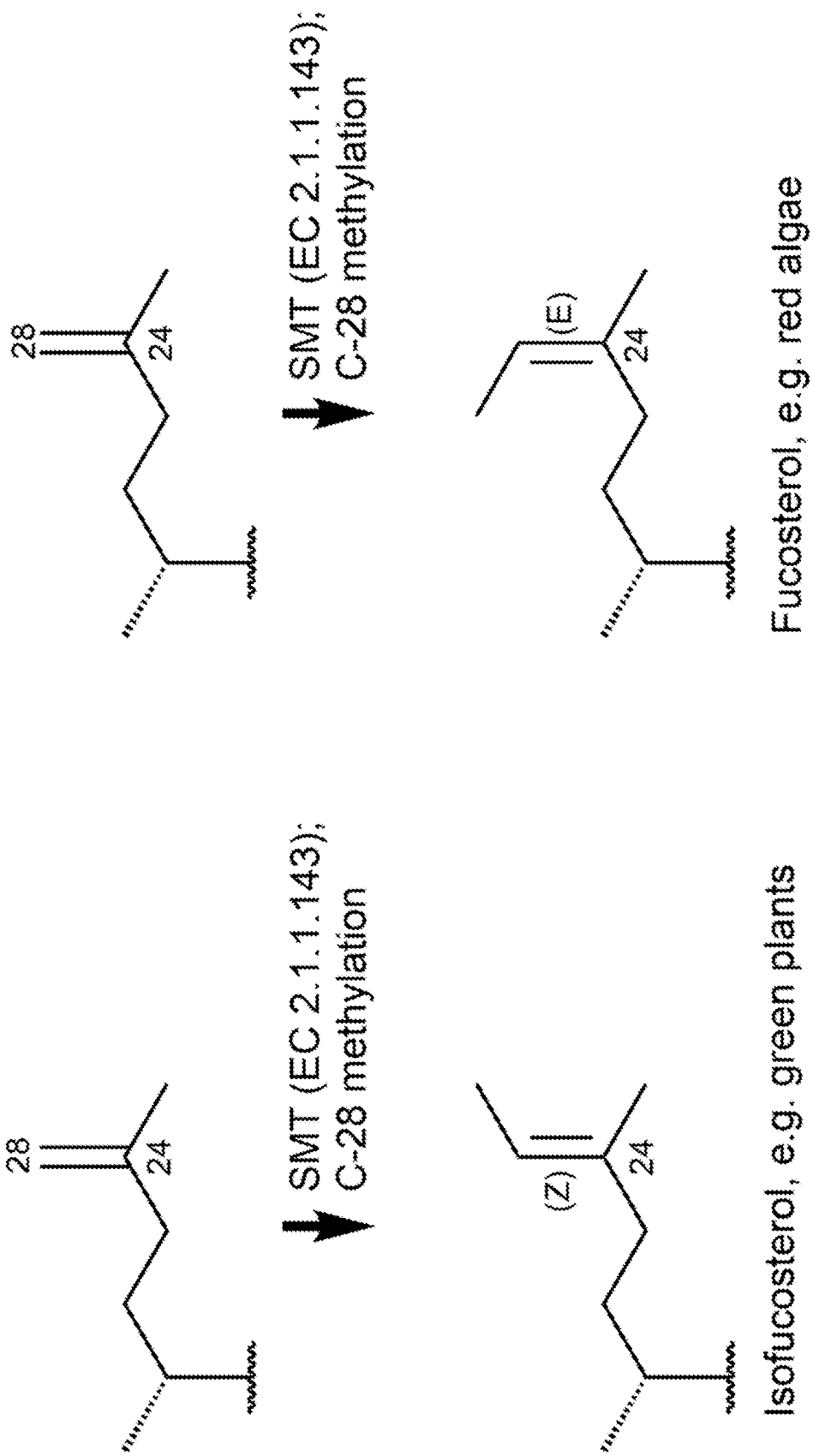

28
24
SMT (EC 2.1.1.143); C-28 methylation
(E)
24
Fucosterol, e.g. red algae
28
24
SMT (EC 2.1.1.143); C-28 methylation
(Z)
24
Isofucosterol, e.g. green plants

Figure 17A

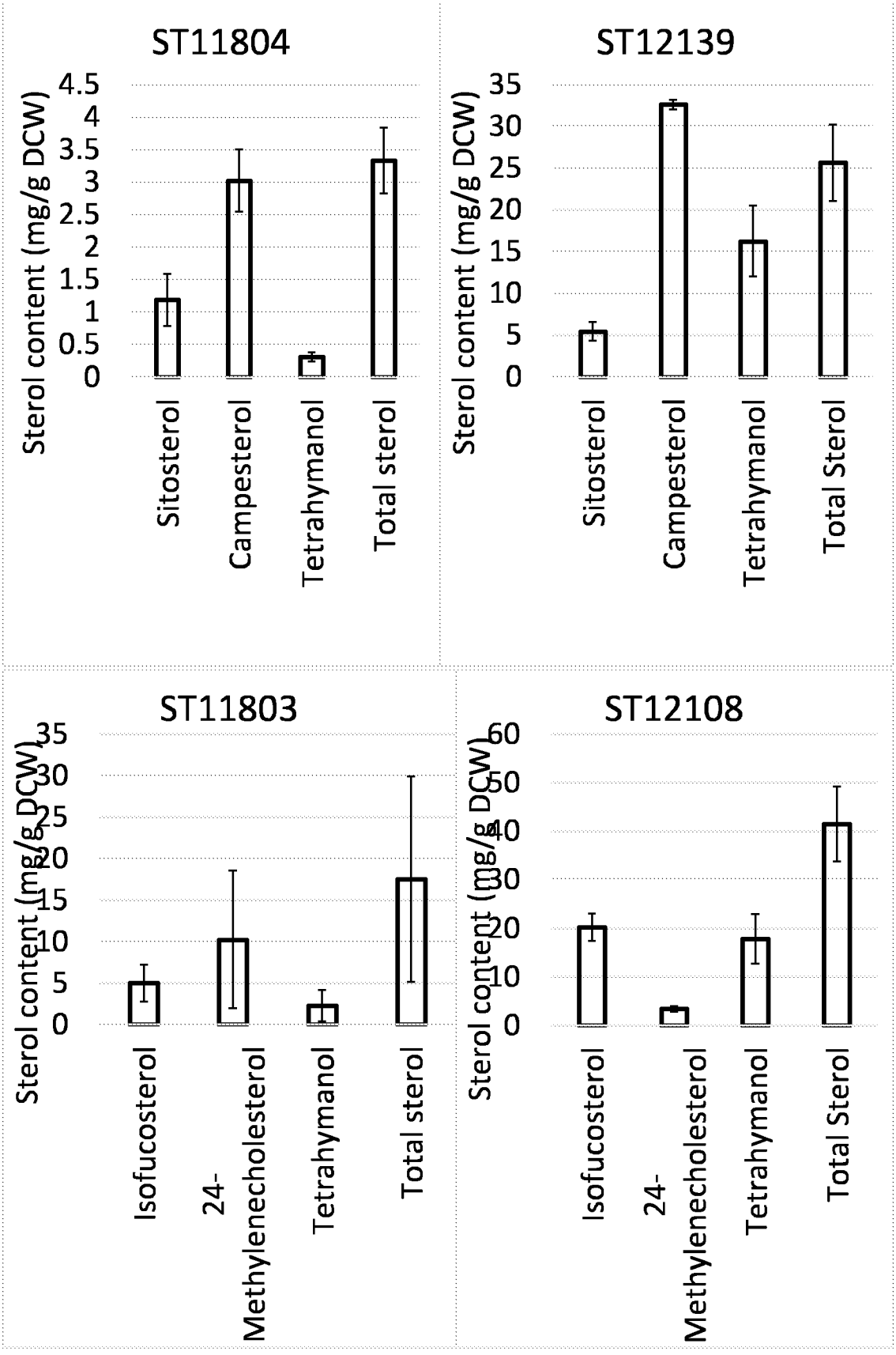

ST11804
Sterol content (mg/g DCW)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Sitosterol
Campesterol
Tetrahymanol
Total sterol
ST12139
Sterol content (mg/g DCW)
35
30
25
20
15
10
5
0
Sitosterol
Campesterol
Tetrahymanol
Total Sterol
ST11803
Sterol content (mg/g DCW)
35
30
25
20
15
10
5
0
Isofucosterol
24-Methylenecholesterol
Tetrahymanol
Total sterol
ST12108
Sterol content (mg/g DCW)
60
50
40
30
20
10
0
Isofucosterol
24-Methylenecholesterol
Tetrahymanol
Total Sterol

Figure 17B

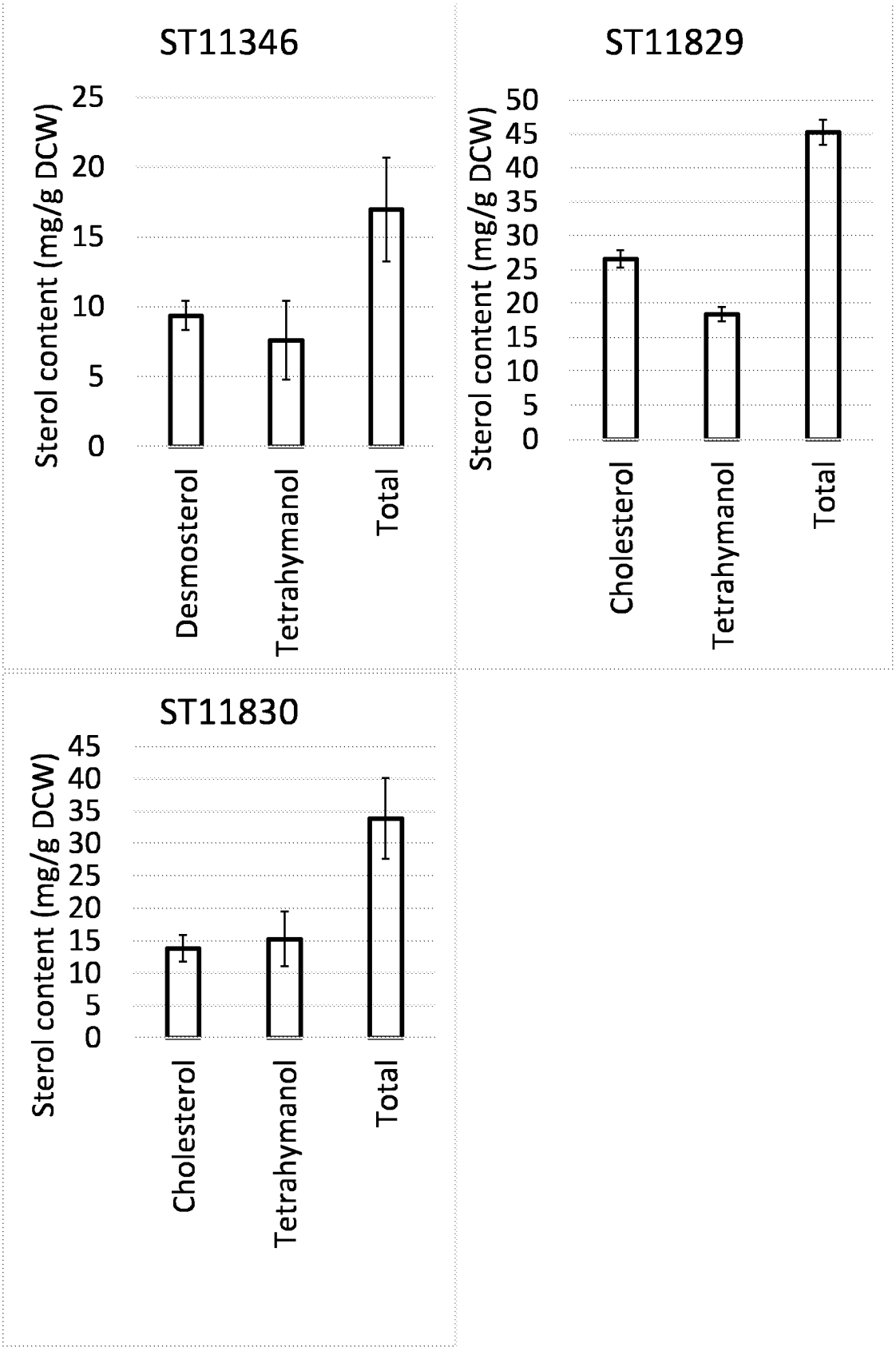

ST11346
Sterol content (mg/g DCW)
25
20
15
10
5
0
Desmosterol
Tetrahymanol
Total
ST11829
Sterol content (mg/g DCW)
50
45
40
35
30
25
20
15
10
5
0
Cholesterol
Tetrahymanol
Total
ST11830
Sterol content (mg/g DCW)
45
40
35
30
25
20
15
10
5
0
Cholesterol
Tetrahymanol
Total

Figure 18A

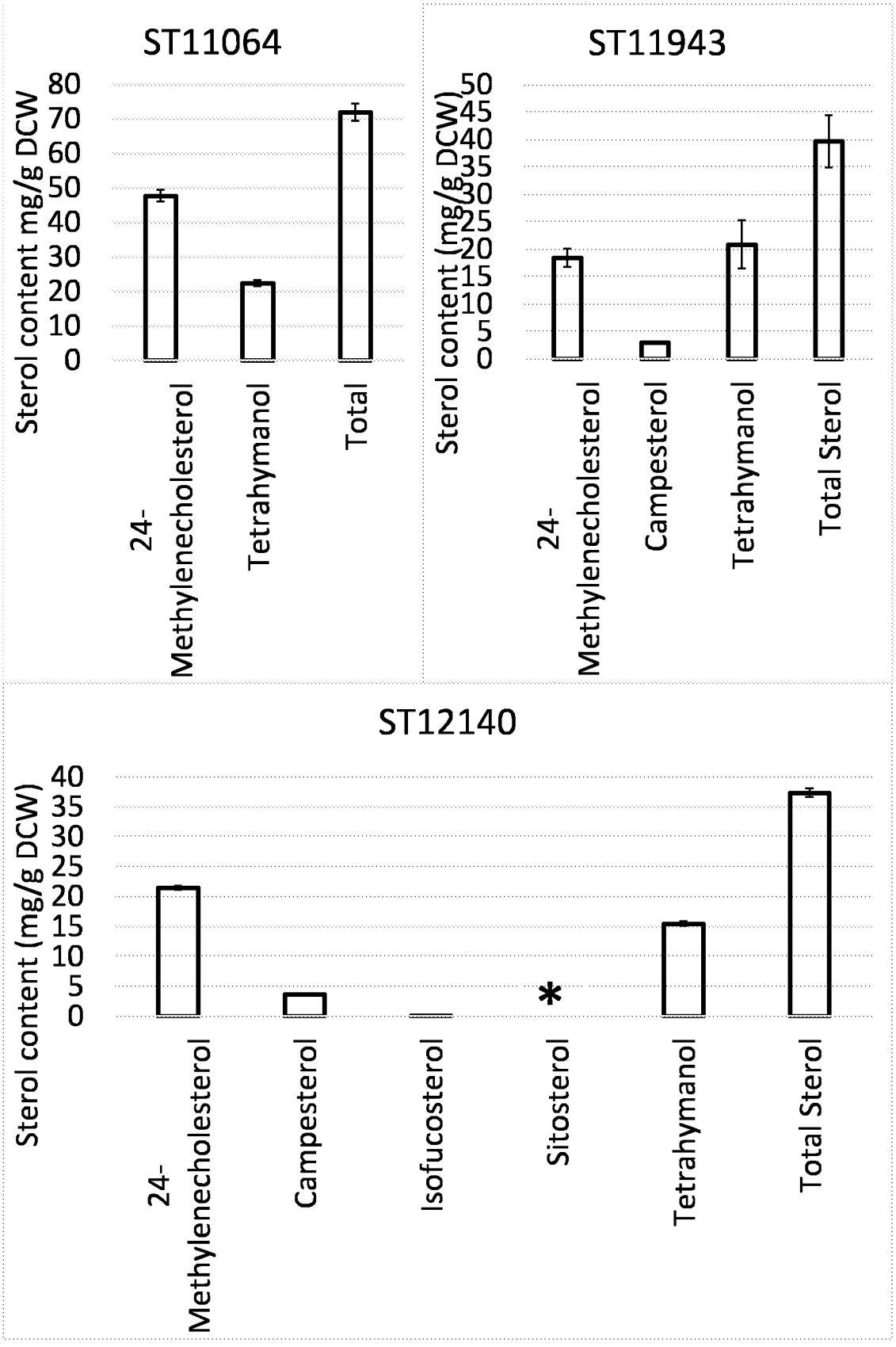

ST11064
Sterol content mg/g DCW
80
70
60
50
40
30
20
10
0
24-Methylenecholesterol
Tetrahymanol
Total
ST11943
Sterol content (mg/g DCW)
50
45
40
35
30
25
20
15
10
5
0
24-Methylenecholesterol
Campesterol
Tetrahymanol
Total Sterol
ST12140
Sterol content (mg/g DCW)
40
35
30
25
20
15
10
5
0
24-Methylenecholesterol
Campesterol
Isofucosterol
Sitosterol
*
Tetrahymanol
Total Sterol

Figure 18B

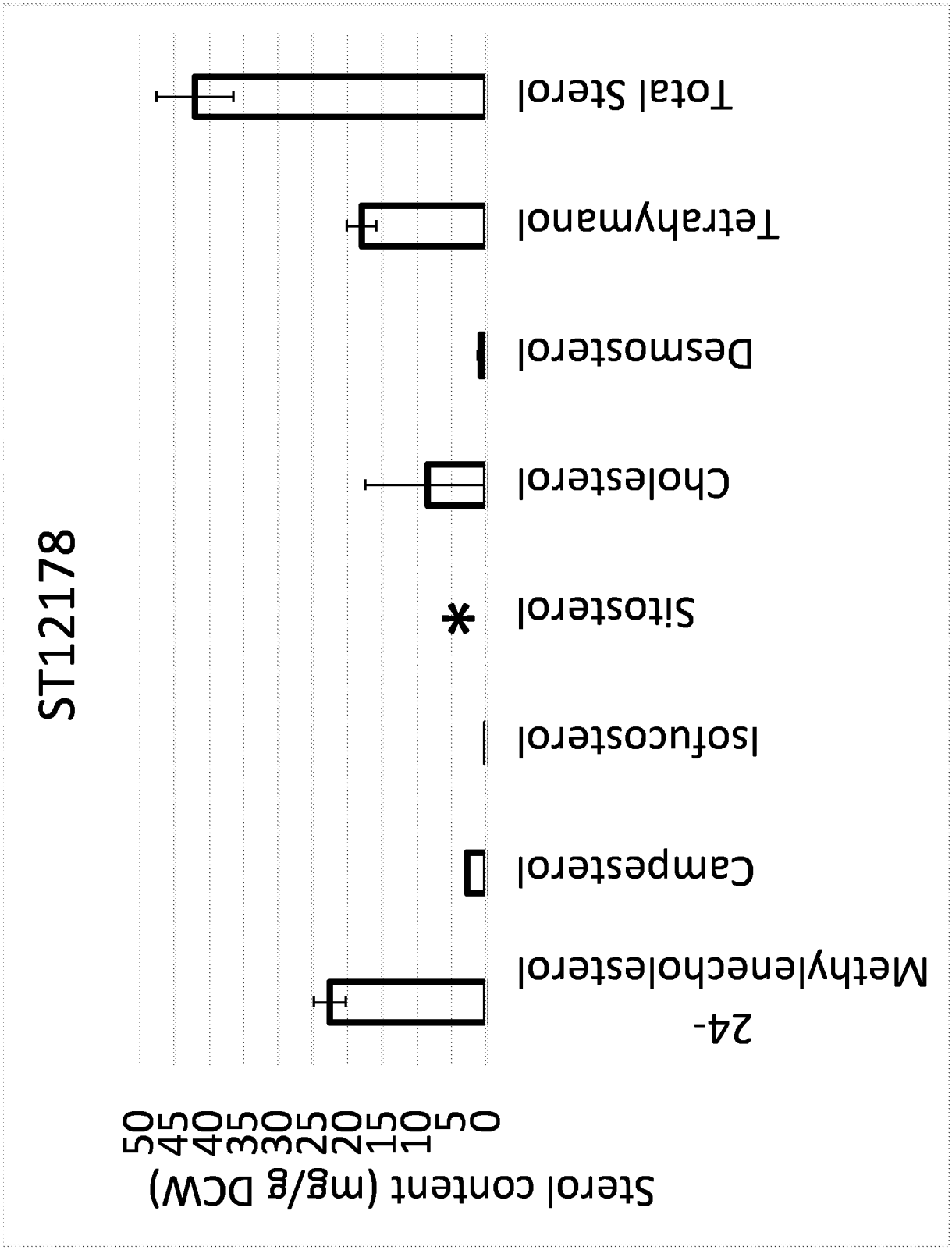

ST121178
Sterol content (mg/g DCW)
0
5
10
15
20
25
30
35
40
45
50
24-Methylenecholesterol
Campesterol
Isofucosterol
Sitosterol
Cholesterol
Desmosterol
Tetrahymanol
Total Sterol

Figure 19

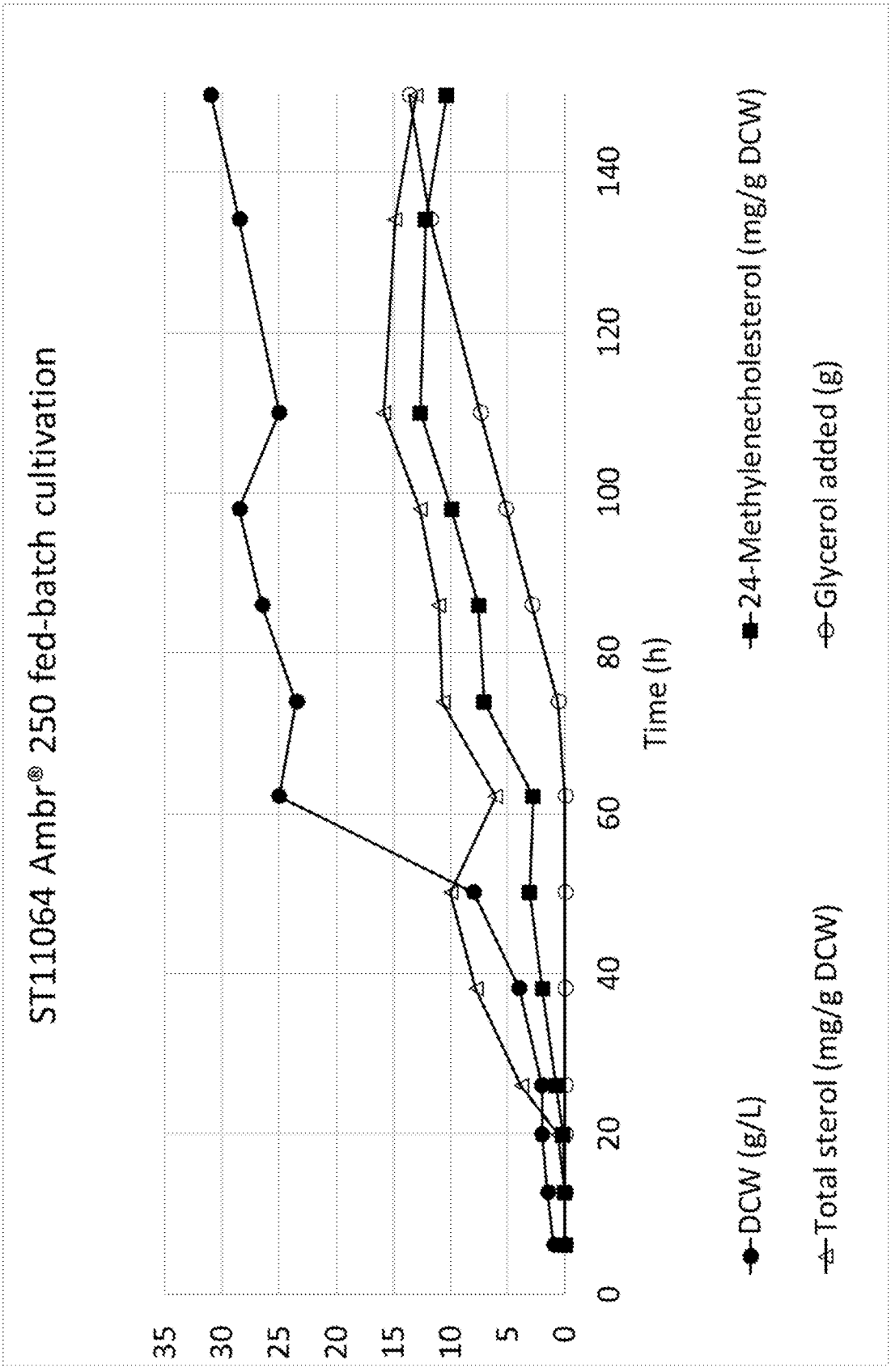

ST11064 Ambr® 250 fed-batch cultivation
Time (h)
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
35
DCW (g/L)
Total sterol (mg/g DCW)
24-Methylenecholesterol (mg/g DCW)
Glycerol added (g)

Figure 20

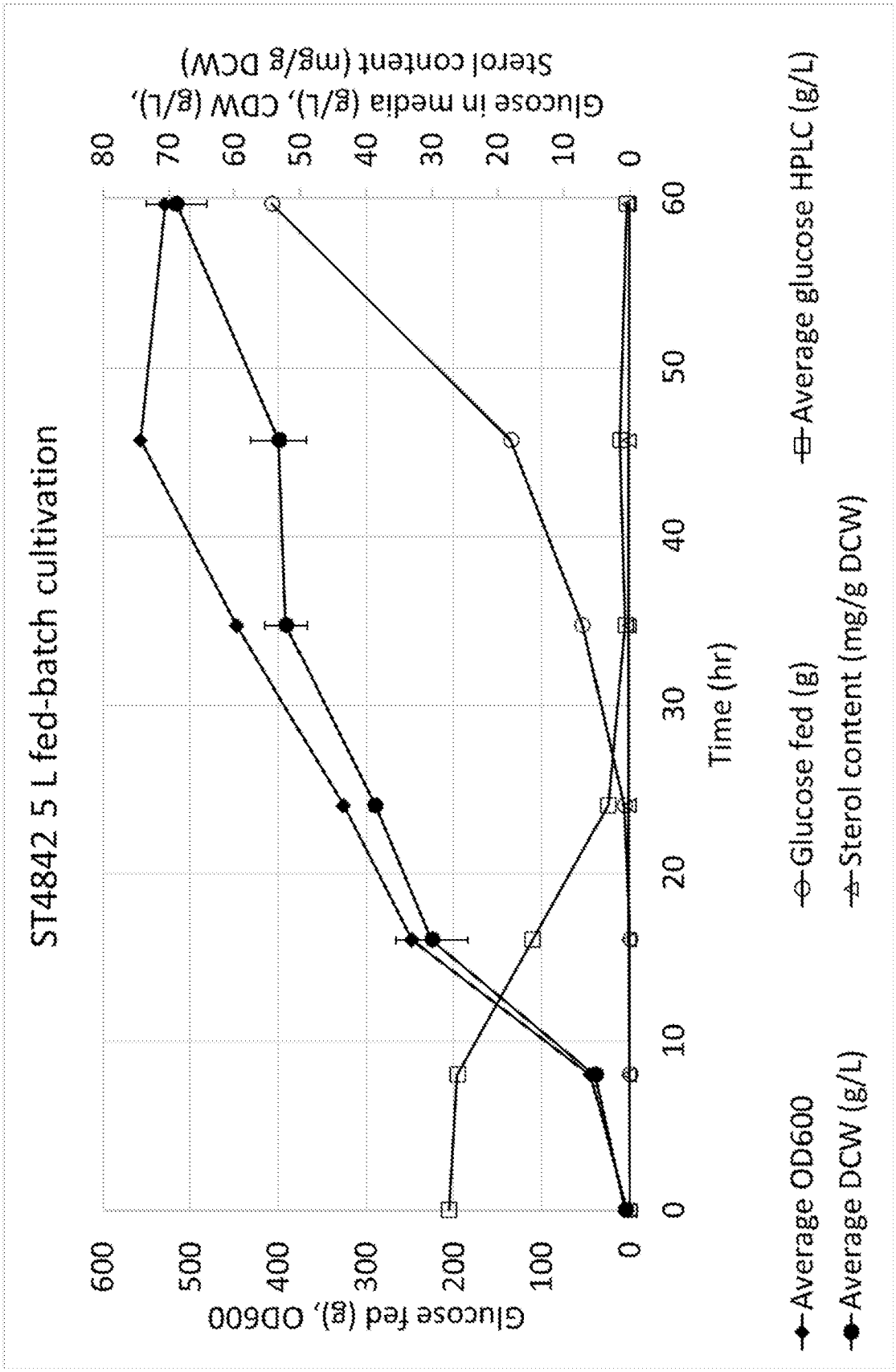

ST4842 5 L fed-batch cultivation
Glucose fed (g), OD600
Glucose in media (g/L), CDW (g/L), Sterol content (mg/g DCW)
Time (hr)
Average OD600
Average DCW (g/L)
Glucose fed (g)
Sterol content (mg/g DCW)
Average glucose HPLC (g/L)

Figure 21

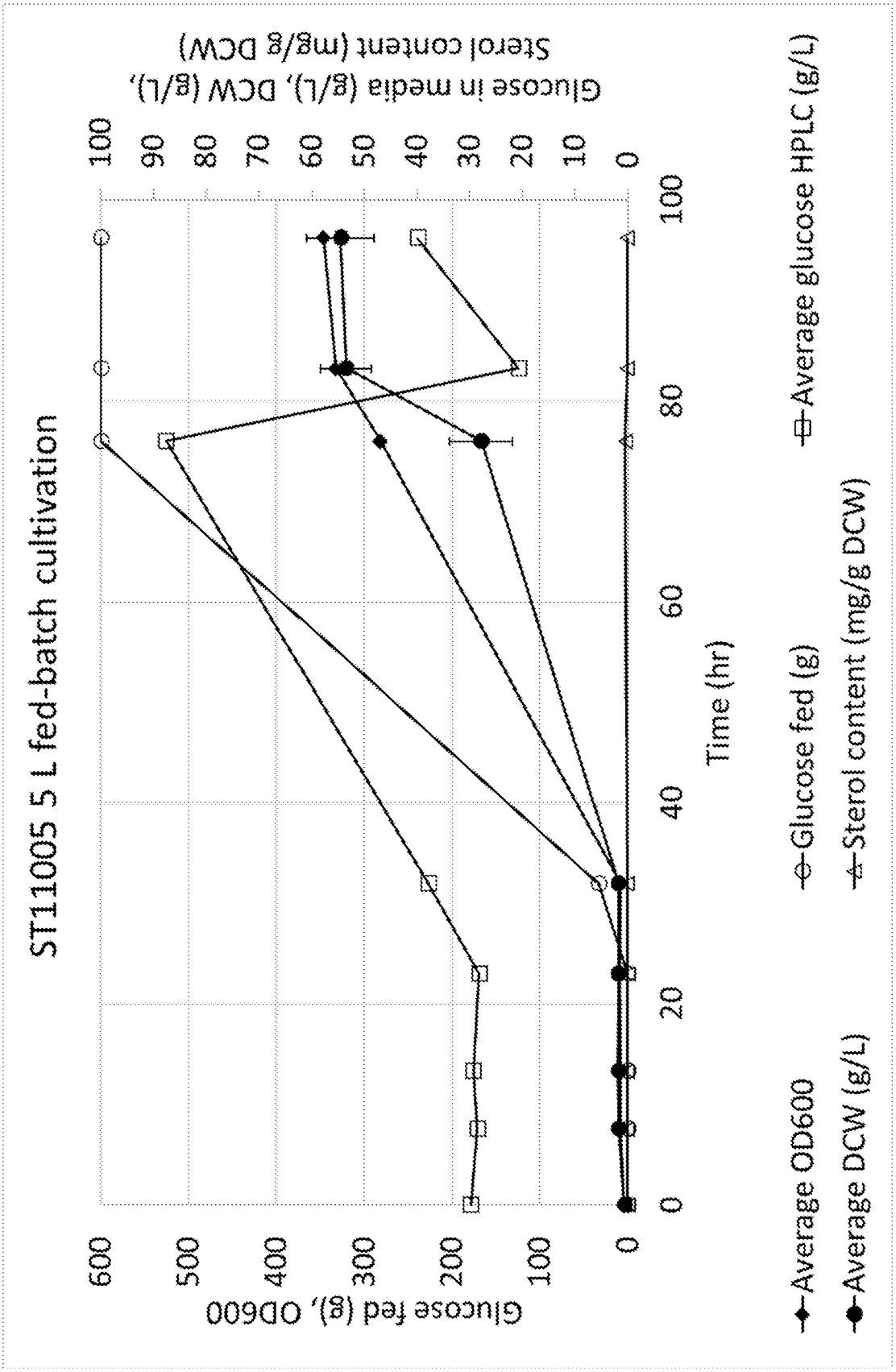

ST11005 5 L fed-batch cultivation
Glucose fed (g), OD600
Glucose in media (g/L), DCW (g/L), Sterol content (mg/g DCW)
Time (hr)
0
100
200
300
400
500
600
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
Average OD600
Average DCW (g/L)
Glucose fed (g)
Sterol content (mg/g DCW)
Average glucose HPLC (g/L)

Figure 22

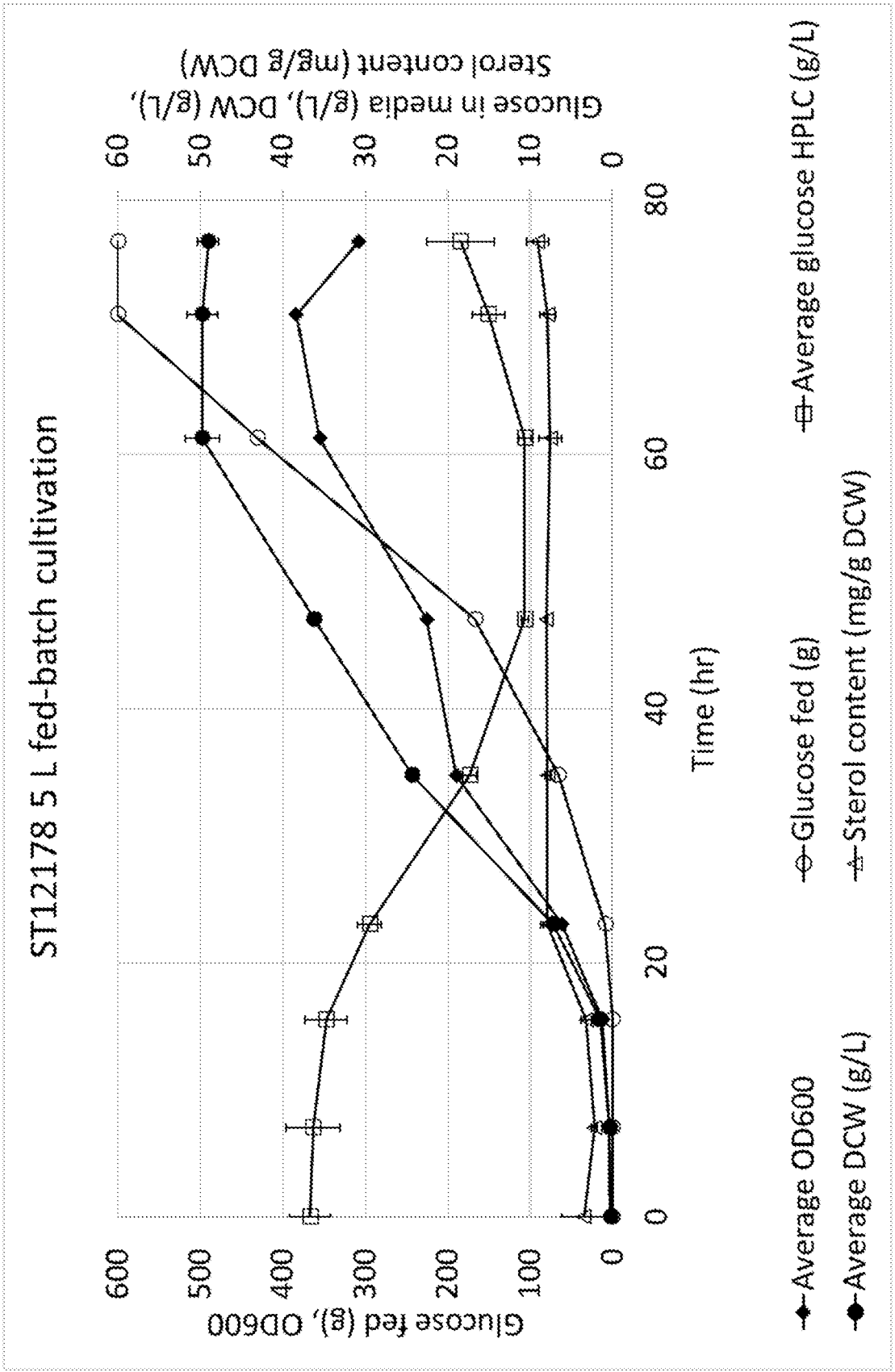

ST12178 5 L fed-batch cultivation
Glucose fed (g), OD600
Glucose in media (g/L), DCW (g/L),
Sterol content (mg/g DCW)
Time (hr)
Average OD600
Average DCW (g/L)
Glucose fed (g)
Sterol content (mg/g DCW)
Average glucose HPLC (g/L)

Figure 23

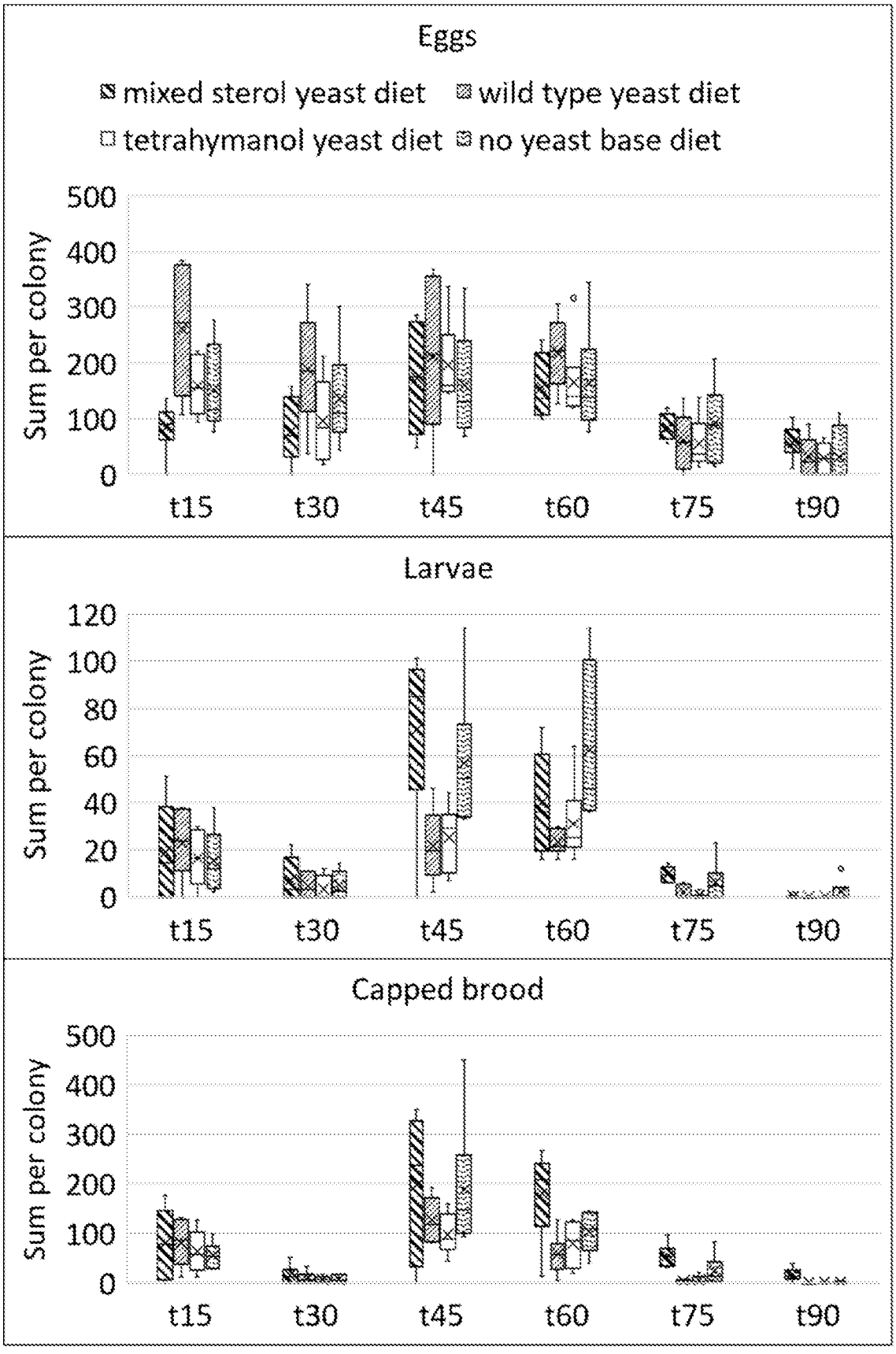
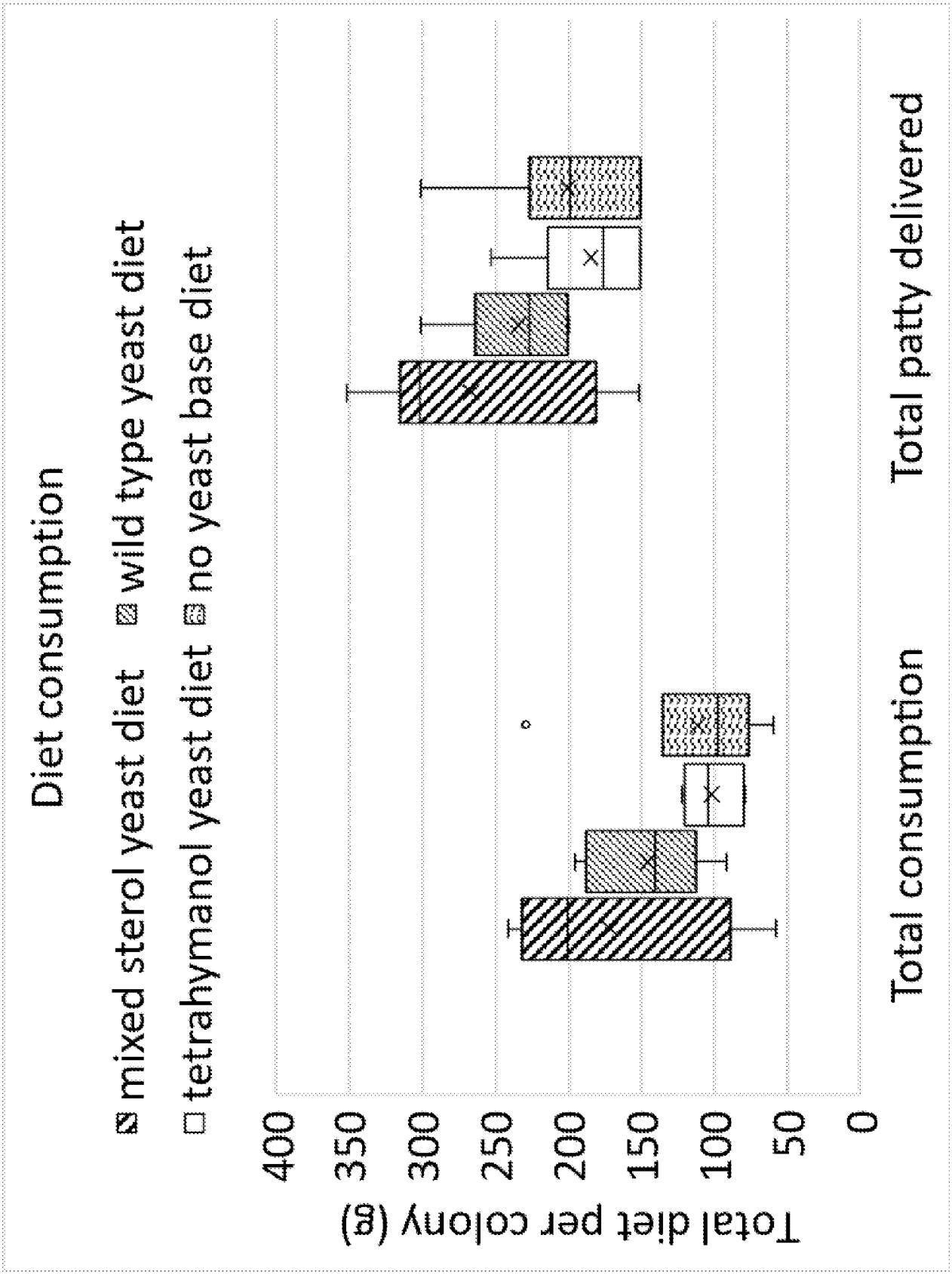

Eggs
mixed sterol yeast diet
wild type yeast diet
tetrahymanol yeast diet
no yeast base diet
Sum per colony
500
400
300
200
100
0
t15
t30
t45
t60
t75
t90
Larvae
Sum per colony
120
100
80
60
40
20
0
t15
t30
t45
t60
t75
t90
Capped brood
Sum per colony
500
400
300
200
100
0
t15
t30
t45
t60
t75
t90

Figure 24


Diet consumption
mixed sterol yeast diet
wild type yeast diet
tetrahymanol yeast diet
no yeast base diet
Total diet per colony (g)
400
350
300
250
200
150
100
50
0
Total consumption
Total patty delivered

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2022/052917

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P33/00 C12N15/52 C12N1/16 C12N9/02 C12N9/10
C12N9/88 C12N9/90 A23K50/90
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12P C12N C12R C11C A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DU, H.-X. ET AL.: "Engineering Yarrowia lipolytica for Campesterol Overproduction", PLOS ONE, vol. 11, no. 1, 11 January 2016 (2016-01-11), pages 1-14, XP093009208, DOI: 10.1371/journal.pone.0146773 the whole document	1-14, 18-37
A	WO 2021/133171 A1 (TECHNISCHE UNIVERSITEIT DELFT) 1 July 2021 (2021-07-01) cited in the application the whole document	1-14, 18-37

-/--

[x] Further documents are listed in the continuation of Box C.
[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 February 2023	25/04/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Fuchs, Ulrike

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2022/052917

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2017/085477 A1 (UNIVERSITY OF NEWCASTLE-UPON-TYNE; UNIVERSITY OF GREENWICH ET AL.) 26 May 2017 (2017-05-26) cited in the application the whole document	1-14, 18-37
E	BE 1 029 373 B1 (APIX BIOSCIENCES) 30 November 2022 (2022-11-30) the whole document	1-8,11, 12, 18-20, 23, 26-28, 31-33

1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2022/052917

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2022/052917

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

1-14(completely); 18-37(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14(completely); 18-37(partially)

- oleaginous yeast for expression of one or more heterologous genes for production of one or more desired non-native sterols or compounds derived therefrom, wherein:
(i) the yeast has reduced production of ergosterol compared with a wild-type oleaginous yeast or is incapable of producing ergosterol, and
(ii) is provided with a sterol surrogate to aid cell growth;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of claim 8(b), claim 8(h), claim 9, claim 11 as dependent on claim 8(h) and claim 9, claim 13, claim 14, claim 18 or claim 19, recovered from a cell culture

---

2. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
a. 24-methylenecholesterol or a derivative thereof, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERGS) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4), and
ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

3. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
b. desmosterol or a derivative thereof, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-24 methyltransferase (ERG6), optionally an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or optionally an attenuated or deleted delta-24 sterol reductase enzyme (ERG4), and
ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

4. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
c. isofucosterol (delta-24(28)-Z isomer) and/or fucosterol (delta-24(28)-E isomer) or a derivative thereof, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted delta-24 sterol reductase enzyme (ERG4),
ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, and
iii. a heterologous gene nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

5. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
d. cholesterol or a derivative thereof, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated or deleted sterol C-24 methyltransferase (ERG6),
ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, and
iii. a heterologous nucleic acid encoding a delta-24 sterol reductase enzyme;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

6. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
e. beta-sitosterol or a derivative thereof, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5),
ii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, and
iii. a heterologous nucleic acid sequence encoding a sterol C-28 methyltransferase enzyme;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

7. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
f. stigmasterol or a derivative thereof, the oleaginous yeast comprising:
i. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme, and
ii. a heterologous nucleic acid encoding a sterol C-28 methyltransferase enzyme, optionally wherein the oleaginous yeast comprises an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and/or delta-24 sterol reductase enzyme (ERG4) and a heterologous nucleic acid encoding a sterol C-22 desaturase enzyme and optionally one or more further heterologous nucleic acid sequences are provided to express a plant delta-24(28) sterol reductase (DWF1) enzyme and/or sterol C-22 desaturase enzyme;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

8. claims: 15, 16, 18-37(all partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
g. a sterol mixture, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5) and an attenuated delta-24 sterol reductase enzyme (ERG4), preferably where the ERG5 gene is deleted and the activity of ERG4 is attenuated by provision of the same encoding sequence, or a corresponding plant delta-24(28) sterol reductase (DWF1) enzyme coding sequence, under the control of a weak promoter selected from PrDGA1 and functionally equivalent weak yeast promoters,
ii. a heterologous nucleic acid encoding a delta-7 sterol reductase enzyme, optionally a heterologous nucleic acid

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

encoding a delta-24(25) sterol reductase and/or optionally additionally a heterologous nucleic acid encoding a C-28 sterol methyltransferase, whereby a non-native sterol mixture can be produced, preferably such that a sterol mixture is produced comprising both 24-methylenecholesterol and campesterol, optionally together with one or more further non-native sterols, e.g. cholesterol;
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19 under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, recovered from a cell culture, preferably wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, recovered from a cell culture

---

9. claims: 17(completely); 15, 16, 18-37(partially)

- oleaginous yeast for production of at least one non-native sterol, wherein the at least one non-native sterol comprises:
h. a sterol mixture, the oleaginous yeast comprising:
i. an attenuated or deleted endogenous sterol C-22 desaturase enzyme (ERG5), prefer. deleted ERG5,
ii. an attenuated delta-24 sterol reductase enzyme (ERG4) or ERG4 substituted by a plant delta-24(28) sterol reductase (DWF1) enzyme providing attenuated delta-24 sterol reductase activity,
iii. a heterologous nucleic acid sequence encoding a delta-7 sterol reductase enzyme,
iv. a heterologous nucleic acid sequence encoding a delta-24(25) sterol reductase, prefer. under the control of the PrGPAT promoter or a functionally equivalent weak promoter,
v. a heterologous nucleic acid sequence encoding a C-28 sterol methyltransferase, prefer. under the control the PrGPAT promoter or a functionally equivalent promoter,
whereby a mixture of non-native sterols can be produced comprising 24-methylenecholesterol, campesterol and one or more further non-native sterols comprising cholesterol, prefer. where 24-methylenecholesterol or campesterol is the dominant sterol of the mixture;
- use of an oleaginous yeast as claimed in claim 8(h), claim 9 or claim 13 which expresses a sterol surrogate as a parent strain to produce an oleaginous yeast according to claim 15(h) or claim 16, where the expression of said sterol surrogate is removed from said parent strain simultaneously with expression of all of a delta-7 sterol reductase, a delta-24(25) sterol reductase and a C-28 sterol

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

methyltransferase plus attenuated ERG4 or substitute plant delta-24(28) sterol reductase enzyme (DWF1);
- method for production of one or more desired non-native sterols which comprises culturing cells of an oleaginous yeast as claimed in any one of claims 7-16, 18 and 19, or obtained by the use of claim 17, under conditions whereby said one or more desired non-native sterols are synthesized;
- artificial dietary composition for bees or other insects or animals which comprises oleaginous yeast cells as claimed in any one of claims 7 to 16, 18 and 19, or obtained by the use of claim 17, recovered from a cell culture, prefer. wherein said yeast cells are yeast cells capable of producing 24-methylenecholesterol or a sterol mixture comprising 24-methylenecholesterol according to any one of 15(a), claim 15(g), claim 15(h), claim 16, claim 18 or claim 19, or obtained by the use of claim 17, recovered from a cell culture

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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